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(54) Title: MONOCYTE CHEMOTACTIC PROTEINS AND RELATED PEPTIDES

(57) Abstract

Disclosed is a homogeneously pure monocyte chemotactic protein, CAP37, and the entire coding sequences for unprocessed and mature human CAP37 protein. Further, the recombinant production, from nucleic acid coding sequences, of mature CAP37 protein and the mature protein with amino-terminal and/or carboxy-terminal extensions is described. Also disclosed are methods to identify and recombinantly produce bioactive peptides derived from the CAP37 protein coding sequence which are effective chemoattractrants of monocytes and/or are capable of binding bacterial lipopolysaccharide. A method of preparing homogeneously pure CAP37 using hydrophobic HPLC is described. Finally, methods of treating wounds and diseased tissue, such as tumors, are described.

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MONOCYTE CHEMOTACTIC PROTEINS AND RELATED PEPTIDES

FEDERALLY SPONSORED RESEARCH

Some aspects of this invention were made in the course of Grants AI 17662 and AI 26589 awarded by the National Institutes of Health and, therefore, the Government has rights in some aspects of this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of pending Application Serial No. 07/375,739, filed July 5, 1989.

FIELD OF THE INVENTION

The present invention relates to methods of making and using a 37,000 dalton cationic granule protein normally synthesized by human polymorphonuclear leukocytes.

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BACKGROUND OF THE INVENTION

In the process of inflammation an initial wave of inflammatory cells, comprised primarily of polymorphonuclear leukocytes (PMN) is soon followed by a second wave of cells, which are predominantly monocytes (Hurley et al.; Wilkinson et al.). Although it is widely held that monocytes arrive at an area of inflammation as a result of chemotaxis (Hayashi et al.), the specific mediator(s) responsible for the recruitment of monocytes has remained unresolved.

Monocytes are derived from pro-monocytes, found in bone marrow. The pro-monocytes differentiate into monocytes which are released into the blood. The monocytes circulate in the blood until they are attracted to the site of injury by the inflammation process. Once monocytes enter into tissue they mature into macrophages, also referred to as mononuclear phagocytes. Macrophages are able to engulf and destroy foreign antigens; accordingly, macrophages play an important role in the body's immunological defense system. The term "monocyte" as used herein and in the claims refers collectively to both circulating monocytes and to macrophages present in tissue.

The preferential migration of monocytes during the latter phase of inflammation indicates the requirement for highly cell-specific chemoattractant, which has little or no

effect on the migration of PMNs. Experiments indicate that a granule-associated cationic protein (mol. wt. 37,000 daltons) from human PMN acts as a monocyte-specific chemoattractant. This protein has been previously referred to in the literature as CAP37, cationic antimicrobial protein of mol. wt. 37,000 daltons. CAP37 protein has been previously shown to (i) bind bacterial lipopolysaccharides with a high degree of specificity and affinity, and (ii) possess antimicrobial activity against a number of Gram negative bacteria (Shafer et al., 1986).

The instant specification discloses a conventional method for purifying CAP37 to homogeneity and a method for making CAP37 using recombinant DNA techniques. The characterization of the cDNA encoding human CAP37 protein is disclosed. The instant specification also discloses proteins and peptides related and/or derived from CAP37 and also having monocyte chemotactic activity. Recombinant DNA techniques for making the proteins and peptides are also disclosed. Finally, the instant specification discloses methods for treating diseases and wounds using CAP37 and its related proteins and peptides.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a recombinant DNA molecule encoding a cationic granule protein having the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly

Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro.

The protein of the present invention is a potent chemoattractant for monocytes and is capable of binding bacterial lipopolysaccharide.

The invention further includes DNA molecules encoding amino terminal extensions of the protein such as, (a) Met Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu Leu Ala Ser Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp, and (b) Met. The invention also includes DNA molecules encoding carboxy terminal extensions such as Gly Pro Ala.

Another important object of the present invention is to provide a recombinant method of producing the cationic granule proteins encoded by the above described sequences. These proteins include the above described amino terminal extensions as well as other modifications, such as the attachment, to the amino or the carboxy terminus of the sequence, of a second protein coding sequence. One example of such a second protein coding sequence is the ricin A chain. Other examples include abrin A chain and trichosanthin. One method of recombinant production is the expression of the cationic granule protein coding sequence fused to the glutathione-S-transferase protein (Sj26) with an in-frame thrombin cleavage site separating the two coding sequences. The fusion protein is then isolated by affinity chromatography for the Sj26 protein portion of the fusion protein, and the cationic granule protein is liberated from the fusion protein by digestion of the fusion protein with the thrombin proteinase.

A further object of the present invention is to provide bioactive peptides having at least 5 amino acids derived from the above described sequence. These bioactive peptides

are either chemotactic for monocytes and/or able to bind bacterial lipopolysaccharide. The present invention further provides a recombinant process for the production of the bioactive peptides.

Another object of the present invention is to provide expression vectors containing DNA sequences encoding any of the above protein sequences. These vectors are useful for introducing the coding sequences into suitable hosts. The expression vectors are effective to express the recombinant proteins in a variety of hosts, for example, bacteria, yeast, and mammalian cells. One vector useful for expression of the above described cationic granule protein contains a cytomegalo virus promoter and a polyadenylation signal from SV40, in addition to the dihydrofolate reductase gene to facilitate selection of the vector in the transformed host.

The invention further includes a method of treating a wound by the application of a topical medication containing a cationic granule protein, having the above described sequence, in the medication in a pharmacologically effective amount to promote wound healing. Other additions to the medication may be desirable such as the inclusion of epidermal growth factor also present in a pharmacologically effective amount to promote wound healing. The topical medication may take any number of standard forms such as pastes, gels, creams, and ointments. Additionally, the invention includes methods of treating tumors and other diseases using CAP37 and its related proteins and peptides.

The invention also includes hybrid proteins containing the cationic granule protein, having the above described sequence, covalently attached to a second protein molecule, such as an antibody, ricin A chain, abrin A chain or trichosanthin.

These and other features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a plot of the data which demonstrates the chemotactic properties of the CAP37 protein.

Fig. 2 illustrates the pcDNAI cloning vector used for subcloning inserts from original lambda clones.

Fig. 3 shows a hydropathy index computation for the entire coding sequence of the CAP37 protein including: the amino-terminal extension and the sequence encoding the mature protein.

Fig. 4 illustrates the expression vectors used for expression of CAP37 protein in mammalian cells.

Fig. 5 depicts the elution profile of the eluates from the hydrophobic HPLC of the impure product obtained after separation on a SEPHADEX G-75 chromatographic column.

Table 1 shows the sequences of peptide fragments derived from the mature CAP37 protein as relevant to the Examples.

Table 2 shows a comparison of N-terminal amino acid sequence of CAP37 protein with several inflammatory cell proteases.

Table 3 shows the nucleic acid and protein sequences of the first isolated cDNA insert having homology to CAP37 protein.

Table 4 shows the nucleic acid and protein sequences of the 6a.1 clone insert.

Table 5 shows the amino acid sequences for three (3) CAP37 peptide fragments which do not share homology with elastase or cathepsin G.

Table 6 illustrates the effect of peptide No. 1 on monocyte chemotaxis.

Table 7 illustrates the effect of peptide No. 3 on monocyte chemotaxis.

DETAILED DESCRIPTION OF THE INVENTION

I. Purification and Characterization of the Human CAP37 Protein

A. Purification and Sequencing of CAP37 Protein.

CAP37 protein was purified from polymorphonuclear leukocytes (PMN) obtained from healthy adult donors (Example 1-A). The crude preparation generated from PMNs was dialyzed and applied to an ion exchange column. The eluent containing the CAP37 protein came off of the column at NaCl concentrations in the range of 0.6 to 0.7 M, as judged by SDS-PAGE of the fractions. This eluent was dialyzed and concentrated. The concentrated preparation was size fractionated and the fractions analyzed by SDS-PAGE to determine the fractions containing the CAP37 protein (Example 1).

Ascites fluid containing antibodies against CAP37 protein was prepared by injecting mice with alum-adsorbed CAP37 protein emulsified in Freund's complete and Freund's incomplete adjuvant (Example 1-C). The specificity of the ascites fluid reactivity against the CAP37 protein was demonstrated in ELISA assays using CAP37, CAP57 (a cationic antimicrobial protein of mol. wt. 57,000 Da; Spitznagel et al.), lactoferrin, myeloperoxidase, cathepsin G and lysozyme as the antigens.

Immunocytochemical analysis of normal human PMN using the monospecific polyvalent mouse antiserum to CAP37 established that CAP37 was a component of the cytoplasmic granules of human PMNs. All other peripheral blood cells which included eosinophils, monocytes, lymphocytes and red blood cells, did not stain positive for CAP37. Immunocytochemical analysis also demonstrated the presence of CAP37 in normal bone marrow, in cells belonging to the myeloid lineage, and in peripheral blood PMN from patients with chronic myelogenous leukemia (CML).

For chemotactic assays the CAP37 protein was further purified by fractionation of the preparation using hydrophobic high performance liquid chromatography (HPLC). For amino acid sequence determination a final desalting step was added utilizing reverse-phase HPLC (Example 1).

The N-terminal amino acid sequence of the CAP37 protein was determined by standard microsequencing procedures (Example 1-D). Table 1 (Example 1) shows the peptide fragment sequences which were determined for part of the mature CAP37 protein. The first 42 amino acids at the amino terminus of CAP37 have received accession number A33070 from the Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C. 20007.

B. Chemotactic Properties of CAP37 Protein

Chemotaxis assays were performed using the modified Boyden chamber technique (Example 2). The results (Fig. 1) demonstrated that CAP37 was obviously chemotactic for human monocytes in the range of 10 to 10,000 pM. CAP37 appeared to be as effective as FMLP in attracting monocytes. These experiments suggest CAP37 did not have any effect on PMN and lymphocyte chemotaxis.

In addition to the observed chemotactic effect on human monocytes, CAP37 at a higher concentration of 1000 ng/ml (2.7×10^{-8} M) was chemotactic for rabbit monocytes as well. This may reflect either (i) a reduction in the numbers of receptors for CAP37 on rabbit monocytes as compared to the number of receptors on human monocytes, or, (ii) a difference in the K_a values for the two species. The effect was selective for monocytes since, rabbit PMN did not show a chemotactic response towards CAP37.

One of the important aspects of identification of a chemotaxin is to distinguish directed cell movement (chemotaxis) through the filters as opposed to merely accelerated random motion (chemokinesis). Using the checkerboard assay (Example 2) it was demonstrated that, in addition to its chemotactic properties, CAP37 protein also has some chemokinetic effect on monocytes.

C. Protein Sequence Comparisons with the Amino Terminus of the CAP37 Protein.

Using the amino terminal sequence of the CAP37 protein, a homology search was conducted of known protein sequences. This search revealed substantial homologies with the amino termini of a subset of serine proteases which mediate a number of functions involved in the inflammatory response (Table 2).

Most of the serine proteases with which CAP37 protein shares its greatest homology (elastase, cathepsin G, rat mast cell proteases I and II, H Factor and cytotoxic T cell specific protein I) are derived from the granules of peripheral blood cells. Further, these homologous proteins are known to play important roles in inflammation, such as cytolysis or degradation of extracellular matrices.

Two other serine proteases, bovine thrombin (Bing et al.) and a trypsin-like protease in guinea pig plasma (Kawaguchi et al.), have been described which exhibit chemotactic activity. Further, Wright et al. have described the presence of a factor(s) in PMN specific granules which acts on serum to produce chemotactically active C5a and the opsonin C3b.

The experiments performed in support of the present invention show the isolation, characterization and purification to homogeneity of a PMN-granule associated protein which has monocyte specific chemotactic activity.

Further, the CAP37 protein is capable of bactericidal activity and thus may perform two very important functional roles in vivo.

II. Cloning of the CAP37 Protein Coding Sequence: Isolation of a First cDNA Having Homology to CAP37 Protein.

For the isolation of the CAP37 cDNA coding sequence, poly-A+ RNA was isolated from tumor cell line HL60 (Example 4-A,B). Using the CAP37 protein specific antibodies generated in Example 1 the HL60 cell line was shown to contain antigens which reacted with this antibody. Double-stranded (ds) cDNA was generated from the isolated RNA molecules (Example 4-C,D). These ds molecules were cloned into the vector lambda-gt10 (Example 4-E).

Plaques resulting from recombinant phage were transferred to duplicate filters and the filters hybridized with two CAP37 sequence specific probes (Example 4-E). The probes were non-coding strand 20-mer degenerative sets generated from the known amino terminal protein sequence of CAP37 protein (Table 1, Example 1) based on the use of preferred human codons (Maruyama et al.). The filters where then probed with each probe set. The duplicate filters showed one recombinant plaque that was convincingly positive with both probes.

The phage was purified from the plaque which tested positive and the double-stranded cDNA insert was isolated from the phage. This insert was then cloned into vector

pcDNAI (Example 5, Fig. 2) and the insert sequenced. The complete sequence of this first cDNA clone is shown in Table 3.

The nucleic acid sequence of the cDNA was translated into the corresponding protein sequences in all three frames. One of the reading frames showed significant alignment to the CAP37 protein sequence with the following exceptions: (i) the cloned sequences indicate that the encoded protein most likely has a precursor form since the cloned sequences contain an additional 48 amino acids 5' to and in frame with the open reading corresponding to the CAP37 protein sequence (Example 6); (ii) there appeared to be some carboxy terminal differences in protein sequence; and, (iii) 19 amino acids present in a CAP37 protein fragment amino acid sequence were not found in the nucleic acid insert sequence (Example 6). The 19 amino acids missing from the protein encoded by the first cDNA clone suggested that although this clone contained coding sequences for the majority of the CAP37 protein it was not the correct cDNA.

Hybridization of other CAP37 specific probes to the above filters did not result in the identification of any more likely candidate plaques.

In order to clone a cDNA encoding the complete CAP37 protein sequence the following strategy was employed. A specific DNA fragment, which would span the region where the discrepancy between the cDNA sequence and the protein

sequence was observed (Example 6), was amplified (Example 7). One end of this DNA fragment was chosen to correspond to the amino acid sequence CQVAGWG (underlined in the CAP37 peptide sequence presented in Example 6): a degenerate 20-mer oligonucleotide probe (designated Intr1c) was designed to correspond to these amino acids.

The second end of the above discussed DNA fragment was less stringently designated. A number of non-degenerate 20-mer primers were designed from the sequence of the non-coding strand of CAP37 first cDNA which was 3' to the end of the site where the nucleic acid sequence diverged from the protein coding sequence (see * in the CAP37 first cDNA sequence in Example 6).

Total human genomic DNA was used as template in DNA amplification reactions utilizing the above described primers (Example 7). The products of the amplification reactions were split into two samples, fractionated on an agarose gel and then one-half of the gel containing a complete set of samples was transferred to a nitrocellulose filter. The filter was then probed with the radioactively-labeled CAP37 first cDNA: one positive band was detected. The DNA was extracted from the region of the second half of the agarose gel corresponding to the positive band. This DNA fragment (Intr1c/669nc) was then sequenced (Example 7-A).

A 50-mer probe corresponding to a region of the Intr1c/669nc sequence which was not present in the CAP37 first cDNA clone was synthesized. This probe was then used to screen 200,000 plaques generated from the lambda-HL60 cDNA library (Example 7-B). After a secondary screen 5 plaques remained clearly positive. One of these clones, designated 6a.1, was sequenced using dideoxy sequencing. The complete DNA sequence of the 6a.1 clone is illustrated in Table 4.

The DNA sequence corresponding to clone 6a.1 was translated in all three reading frames. One reading frame aligned with the amino acid sequences of the CAP37 peptide fragments determined in Example 1. The CAP37 mature protein coding sequence was preceded, in frame, by 26 amino acids, and had a short carboxy terminal extension. These results indicate that the 6a.1 clone is a complete cDNA clone encoding the CAP37 protein.

The first approximately 20 amino acids of the cDNA encoded CAP37 protein have a high degree of hydrophobicity (Fig. 3). This sequence has the length and hydrophobicity of a typical eucaryotic secretory signal sequence (Example 8). The remaining amino acids in this 26 amino acid sequence and/or the carboxy-terminal extension may be involved in further cellular processing of the CAP37 protein or they may represent an inactive precursor form of the protein.

The portion of the open reading frame corresponding to the mature form of the CAP37 protein has an estimated molecular weight of 24,276 kilodaltons. Previously Shafer et al. (1986) determined the molecular weight of the CAP37 protein to be approximately 37,000 daltons. This discrepancy is most likely the result of post-translational modification, e.g. glycosylation, of the CAP37 protein.

Subsequent to the isolation and sequencing of the 6a.1 cDNA insert, the complete protein sequence of the mature CAP37 protein was determined. With the exception of the above described amino and carboxy terminal extensions, the coding sequence of the mature CAP37 protein exactly aligns with the protein sequence encoded by the 6a.1 insert.

III. Recombinant Expression of the CAP37 Protein

Factors that are involved in successful expression of a cloned gene in a particular system include, solubility within the cell, non-toxicity to the cell, possible secretion by the expressing cell, low levels of proteolytic digestion by the cell and ease of purification in the system. Folding, disulfide bond formation, as well as post-translational modifications (glycosylation, phosphorylation, etc.) can affect expression and synthesis of proteins; these factors differ according to the host cells used for expression.

The E. coli plasmid system to express CAP37 was described by Smith et al. This system involves the use of a plasmid expression vector designed to direct synthesis of foreign polypeptides as fusions with the protein Sj26; Sj26 is a glutathione S-transferase protein from Schistosoma japonicum. Purification of the fusion protein is accomplished by affinity chromatography on immobilized glutathione. Smith et al. have described the use of expression vectors encoding a thrombin cleavage site immediately adjacent the carboxy terminus of Sj26 and the amino-terminal of the foreign protein which is to be expressed. Recovery of the foreign protein can be achieved by thrombin digestion of the purified fusion protein (Example 9-A). The thrombin digestion is followed by a final purification step to remove Sj26. High yields of several soluble proteins have been demonstrated with these plasmids.

Alternatively, the thrombin cleavage site can be replaced by another protease cleavage site such as collagenase (EC 3.4.24.3, from Clostridium histolyticum) by insertion of an oligonucleotide linker containing the appropriate cleavage sequence (e.g., see Example 10-B).

In addition to the above described vector there are numerous E. coli expression vectors known to one of ordinary skill in the art useful for the expression of CAP37 protein. If necessary an amino terminal methionine can be provided the CAP37 protein coding sequence by insertion of a Met

codon 5' and in-frame with the CAP37 protein coding sequence. Also, the carboxy-terminal extension of the cDNA encoded CAP37 protein can be removed using standard oligonucleotide mutagensis procedures.

There are several advantages to yeast expression systems. First, evidence exists that proteins produced in a yeast secretion systems exhibit correct disulfide pairing (Zsebo et al.). Second, post-translational glycosylation is efficiently carried out by yeast secretory systems (Gillis et al.; Thim et al.; Chang et al.; Hoylaerts et al.; Lemontt et al).

The Saccharomyces cerevisiae pre-pro-alpha-factor leader region (encoded by the MFα-1 gene) is routinely used to direct protein secretion from yeast (Brake et al., 1984). The leader region of pre-pro-alpha-factor contains a signal peptide and a pro-segment which includes a recognition sequence for a yeast protease encoded by the KEX2 gene: this enzyme cleaves the precursor protein on the carboxyl side of a Lys-Arg dipeptide cleavage-signal sequence. The CAP37 protein coding sequence is fused in-frame to the pre-pro-alpha-factor leader region (Example 9-B). This construct is then put under the control of a strong transcription promoter, such as the alcohol dehydrogenase I promoter or a glycolytic promoter. The CAP37 protein coding sequence is followed by a translation termination codon which is followed by transcription termination signals. Alternatively, the CAP37 protein coding sequences can be

fused to a second protein coding sequence, such as Sj26 (Example 9-A) or β-galactosidase (Example 10-B), used to facilitate purification of the fusion protein by affinity chromatography. The insertion of protease cleavage sites to separate the components of the fusion protein, such as described above for CAP37 protein expression in E. coli, is also applicable to constructs used for expression in yeast.

Mammalian cells permit the expression of proteins in an environment that favors important post-translational modifications such as folding and cysteine pairing, addition of complex carbohydrate structures, and secretion of active protein.

Vectors useful for the expression of CAP37 protein in mammalian cells are shown in Fig. 4. These vectors are characterized by insertion of the CAP37 protein coding sequence between a strong viral promoter and a polyadenylation signal. The vectors further contain genes conferring either Genetimicin or methotrexate resistance for use as selectable markers. The CAP37 protein coding sequence has been introduced into a Chinese hamster ovary cell line using a methotrexate resistance-encoding vector (Example 9-C). Presence of the vector DNA in transformed cells has been confirmed by Southern analysis and production of an RNA corresponding to the CAP37 protein coding sequence has been confirmed by Northern analysis.

Alternative vectors for the expression of CAP37 in mammalian cells, are those similar to those developed for the expression of human gamma-interferon, tissue plasminogen activator, clotting Factor VIII, hepatitis B virus surface antigen, protease Nexin1, and eosinophil major basic protein, will be employed (Gray et al; Wood et al.; Patzer et al.; Berman et al.; McGrogan et al, 1988 A and B). Further, the vector shown in Fig. 2 contains CMV promoter sequences and a polyadenylation signal available for expression of inserted DNAs in mammalian cells (such as COS7).

The identification and use in cloning of the entire CAP37 protein coding sequenced identified by cDNA sequencing is particularly useful in mammalian cells since the putative pre-pro sequence (i.e. the first 26 amino acids of the cDNA encoded protein coding sequence) may have important effects on secretion and other protein processing events.

IV. Bioactive Peptide Fragments of the CAP37 Protein

A bioactive portion of the CAP37 protein is defined as a peptide fragment of at least about 5 amino acids derived from the CAP37 protein coding sequence (Table 4) which has chemotactic or lipopolysaccharide-binding properties (Example 11).

One method of identifying bioactive fragments of CAP37 is described in Example 12. Of the three fragments identified and synthesized, two were active. Another method

of identifying bioactive fragments is described in Examples 10 and 11. In this latter method the CAP37 protein coding sequence is fragmented by digestion with DNAse I (Example 10-A). The CAP37 protein coding sequence could also be fragmented using specific restriction endonucleases, such as HaeIII which has 19 cut sites in the coding sequence. These fragments are then ligated into a vector which allows identification of in-frame fusions by a simple plate assay (Example 10-B). The presence of CAP37 protein coding DNA sequences can be verified in the clones utilizing colony hybridization. The in-frame fusions represent candidate CAP37 peptides which can be assayed for their chemotactic and lipopolysaccharide binding properties as described in Example 11.

Glycosylation and other post-translational modifications may not be required to detect these activities, thus expression of these peptides in E. coli is the method of choice. However, the fusions of the bioactive peptides to β-galactosidase can also be expressed in yeast or mammalian cells essentially as described above and in Example 9.

V. Anti-CAP37 Antibodies

In another aspect, the invention includes an antibody which is specific against CAP37 protein or bioactive peptide derivatives thereof, or specific against CAP37 protein/peptide fusion proteins (Examples 9-A, 10, and 11).

To prepare antibodies, a host animal, such as a rabbit, is immunized with the purified CAP37 protein/peptide antigen. The host serum or plasma is collected following an appropriate time interval, and this serum is tested for antibodies specific against the CAP37 protein antigen. Example 12 describes the production of rabbit serum antibodies which are specific against the CAP37 protein/peptide.

The gamma globulin fraction or the IgG antibodies of immunized animals can be obtained, for example, by use of saturated ammonium sulfate or DEAE SEPHADEX, or other techniques known to those skilled in the art for producing polyclonal antibodies.

Alternatively, the purified antigen may be used for producing monoclonal antibodies. Here the spleen or lymphocytes from an immunized animal are removed and immortalized or used to prepare hybridomas by methods known to those skilled in the art.

Antibodies secreted by the immortalized cells are screened to determine the clones that secrete antibodies of the desired specificity (Example 12), for example, using the Western blot method (Ausubel et al.).

VI. Hybrid Proteins Created with CAP37 Protein

In another aspect, the invention includes CAP37 fused at its amino or carboxy end with a second protein or peptide to form a hybrid protein. The CAP37 protein making up the

hybrid protein is preferably recombinantly produced CAP37 protein or a bioactive portion thereof, as described above.

The hybrid CAP37 protein or bioactive peptide (CAP37 protein/peptide) may be formed by chemical conjugation or by recombinant techniques. In the former method, the second peptide and CAP37 protein/peptide are modified by conventional coupling agents for covalent attachment (Duncan; Cumber).

Where CAP37 protein/bioactive peptide is used in tumor therapy, the protein/peptide may be advantageously fused with a polyclonal or monoclonal antibody specifically directed against target tumor-specific cell surface antigen(s). This fusion protein is then infused at or near the tumor site to attract monocytes to the site.

As an alternative chemical conjugation method, CAP37 protein/peptides not containing cysteine may be prepared to contain cysteine to allow disulfide coupling of the CAP37 protein to an activated second protein, thus simplifying the coupling reaction. The expression vector used for production of recombinant CAP37 bioactive peptide can be modified for insertion of an internal or a terminal cysteine codon according to standard methods of site-directed mutagenesis or oligonucleotide linker insertion.

In a preferred method, the hybrid protein is prepared recombinantly using an expression vector in which the coding sequence of the fusion peptide is joined to the CAP37 protein/bioactive peptide coding sequence. For example, an

expression vector formed is designed to contain the following components: (a) a synthetic trp/lac promoter positioned appropriately ahead of a ribosome binding site that is also positioned appropriately ahead of an ATG start codon which is 5' to an cloning site; (b) the ricin A chain coding sequence (Olnes; Olnes et al.; Lamb et al.); (c) a spacer encoding sequence coding for 10 proline residues, which spaces the ricin A chain and CAP37 protein/peptide moieties; (d) the coding sequence for the CAP37 protein/peptide; and, (e) a stop codon positioned adjacent the carboxy-terminal codon of CAP37 protein/peptide. The method generally follows that used in fusing a soluble CD4 to domains 2 and 3 of pseudomonas exotoxin A, as described previously (Chaudhary).

The expression vector containing the fusion protein coding sequences is analyzed for expression of the hybrid protein. Briefly, the expression vector is cultured in a suitable bacterial host under IPTG induction conditions to a desired cell density. The cells are harvested, ruptured by sonication, and the cell material is clarified by centrifugation. The clarified material is tested for (a) chemotactic ability, to confirm CAP37 protein/bioactive peptide activity, and (b) for ribosome inhibition activity, to confirm the ricin A chain enzymatic activity.

The protein may be purified by molecular-sieve and ion-exchange chromatography methods, with additional

purification by polyacrylamide gel electrophoretic separation and/or HPLC chromatography, if necessary.

The ricin/CAP37 fusion protein can be used as a very specific anti-tumor reagent for cancers of monocyte cells.

It will be appreciated from the above how other second protein-CAP37 protein/peptide fusion proteins may be prepared. One variation on the above fusion is to exchange positions of the ricin A chain and CAP37 protein/peptide coding sequences in the fusion protein expression vector. Other variations would use abrin A chain or trichosanthin rather than ricin A chain. (Olnes et al., 1982; Lifson et al., 1989; Chow et al., 1990; Collins et al., 1990).

VII. Utility

The cloning and expression of human CAP37 protein provides an important source of the purified protein -- simplifying preparation and allowing production of large quantities of the protein.

The purified CAP37 protein, or a bioactive peptide thereof, can be applied to wound sites resulting in a two-fold benefit: (i) the protein is a strong anti-microbial agent (Shafer et al., 1986), and (ii) monocytes are attracted to the wound site. The CAP37 protein can be applied at a wound site by infusion, allowing the protein to form a gradient in surrounding tissue by diffusion. Alternatively, the CAP37 protein/peptide can be formulated in a topical medication, such as pastes, gels, creams, and

ointments, by standard methods (e.g., West et al.). The topical medication is then applied at the wound site: this method of application allows a controlled release of the protein at the wound site. Such topical formulations may also contain other components, such as antibiotics or epidermal growth factor (Brown et al.), to facilitate healing.

The purified CAP37 protein, or a bioactive peptide thereof, may also be used in treating other diseases such as cancers. CAP37 can be administered through systemic injection, intravesical administration or may be injected locally into an accessible tumor mass. The route of administration would depend on the nature and size of the tumor or other diseased tissue being treated. CAP37, or a bioactive peptide thereof, could be administered using protocols for adjuvant immunotherapy currently using bacterial vaccines such as Bacille Calmette-Guerin (BCG) or Corynebacterium parvum. Such protocols are well known in the art. (Regelson et al., 1986;; Martinez-Piñeiro et al., 1988; Cumming et al., 1989).

Essentially, an effective amount of CAP37 would be mixed with a suitable solvent or carrier and administered to an animal by one of the routes of administration established for other adjuvants such as BCG. CAP37 could be encapsulated in multilamellar phospholipid vesicles, or other encapsulating material, to aid in administration. Depending on the area to be treated, the route of

administration and the dose, CAP37 might be administered without a carrier.

An effective dose of CAP37 would vary depending upon the route of administration and the area to be treated. CAP37 could be administered systemically or intraperitoneally in a dose of 100 ng/kg.

Because of its ability specifically to attract monocytes, CAP37 could be used to treat any disease involving monocyte localization. These diseases would include neoplastic diseases, rheumatoid arthritis, hepatitis, chromic obstructive pulmonary disease, chronic periodontal disease, parasitic diseases such as malaria, tuberculosis and leprosy. CAP37 could also be used to treat diseases involving defects of monocyte chemotaxis such as Wiscott Aldrich syndrome, chronic mucocutaneous candidiasis, chronic granulomatous disease, Chediak Higashi syndrome, systemic lupus erythematosus and herpes simplex.

Since the CAP37 protein binds bacterial lipopolysaccharide, CAP37 protein, or a bioactive peptide thereof, may also provide an effective treatment for endotoxemia. A solution of the CAP37 protein may be parenterally applied to a patient to bind endotoxins that are circulating in blood and facilitate their clearing.

Further, as discussed above (Section VI), hybrid proteins containing CAP37 protein, or bioactive portions thereof, may be useful in tumor therapies, particularly tumors of monocytes.

The following examples illustrate, but are in no way intended to limit the present invention.

Example 1

Purification and sequencing of human CAP37 protein

A. Source and Preparation of Peripheral Blood PMN and Monocytes

Blood was collected into sterile sodium-EDTA tubes by venepuncture from healthy adult donors. The polymorphonuclear leukocytes (PMN) were separated from mononuclear cells essentially by the ficoll hypaque density gradient technique of Boyum, followed by dextran sedimentation (T500, Pharmacia 3% in saline) and hypotonic lysis of contaminating red blood cells (RBC).

The mononuclear cell band was further purified to separate the monocytes from the lymphocytes. The mononuclear cells were washed once in phosphate buffered saline (PBS: 0.01 M Na_2HPO_4, containing 0.15 M NaCl, pH 7.4) and resuspended in PBS to a total volume of 5.1 ml. This cell suspension was then added to 6.7 ml of SEPRACELL-MN reagent (a colloidal, silica-based medium having a density of 1.099 g/ml, and available from Sepratech Corporation, Oklahoma City, Oklahoma), and centrifuged (1500 g for 20 min at 22°C). The cells were washed twice in PBS (150 X g, 15 min) and resuspended in Geys buffered saline (Gibco) containing 2% bovine serum albumin (BSA - Fraction V, endotoxin free, Boehringer Mannheim Biochemicals) at a final

concentration of 2 x 10^6 cells/ml. (Geys buffer has the following composition: $CaCl_2$(anhyd.) 0.17 g/L; KCl 0.37 g/L; KH_2PO_4 0.03 g/L; $MgCl_2 \cdot 6H_2O$ 0.21 g/L; $MgSO_4 \cdot 7H_2O$ 0.07 g/L; NaCl 7 g/L; $NaHCO_3$ 2.27 g/L; $Na_2HPO_4 \cdot 7H_2O$ 0.226 g/L; D-Glucose 1.00 g/L.) The monocytes were greater than 95% pure as determined by Wright's and non-specific esterase staining.

Peripheral blood mononuclear cells from adult female rabbits, were separated on a 61% PERCOLL (Pharmacia) gradient (Chambers et al.). This technique separated the mononuclear cells from the RBC and PMNs. No further separation of monocytes from lymphocytes was undertaken with the rabbit blood.

B. Purification of CAP37

A granulocyte concentrate (>95% PMN) was obtained by leukophoresis from a normal human donor. The PMN were disrupted by homogenization in a Potter-Elvehjem tissue grinder (Kontes) for 60 sec at 4°C. Mixed (specific and azurophil) granules were harvested by differential centrifugation. The supernatant obtained by centrifuging at 126 x g for 15 minutes was further centrifuged at 20,000 x g for 20 minutes to yield a pellet of mixed granules which was extracted at 4°C with 0.2M sodium acetate (pH 4.0). Granule debris was collected by high-speed centrifugation at 20,000 x g for 30 min. The protein concentration was determined by

the method of Bradford with chick egg white lysozyme as the standard. Bradford et al., 1976.

The crude granule extract was dialyzed against 50 mM sodium acetate (pH=5) and 0.15 M NaCl at 4°C overnight. The dialysate was then applied to a carboxymethyl SEPHADEX ion exchange column (a weakly acidic cation exchanger having sodium as a counter-ion and carboxymethyl as a functional group, and available from Pharmacia, Piscataway NJ) which had been equilibrated with 50 mM sodium acetate (pH=5) and 0.15 M NaCl. The column was extensively washed with 6M urea in 0.05M sodium acetate (pH5) containing 1.5M sodium chloride. Protein bound to the column was eluted using a two-step linear salt gradient consisting of 0.15 to 0.4 M and 0.4 to 1.0 M NaCl in 50 mM sodium acetate pH=5. Protein elution was monitored by measuring the absorbance of the eluent at 280 nm. Salt concentrations of the fractions were determined by conductivity measurements. Fractions from the CMS column were tested by ELISA using antiserum to CAP37 to determine those fractions which contained CAP 37.

The positive fractions were pooled and dialyzed overnight against 0.2 M sodium acetate (pH=4) at 4°C, concentrated by membrane ultrafiltration at 4°C using a YM-5 membrane filter (an ultrafiltration membrane having very low non-specific protein binding properties, a nominal molecular weight cut-off of 5000 daltons, a clean water flow rate of 0.07 -0.1ml/min/cm^2 and available from Amicon Corp., Lexington KY). The concentrated preparation was applied to

a SEPHADEX G-75 SF (Pharmacia) column (0.5 by 50 cm) which had been equilibrated with 0.2 M sodium acetate (pH=4). (SEPHADEX G-75 is a chromatographic medium comprised of a bead-formed gel prepared by cross-linking dextran with epichlorohydrin; said medium having a dry bead size of 10-40 μ, bed volume of 12-15 ml/g of dry SEPHADEX, and fractionation range for globular proteins and peptides of 3,000-70,000 daltons.) Fractions were eluted using the equilibration buffer and the A_{210} of each fraction determined. The fractions were analyzed by ELISA and by SDS-PAGE to determine the fraction containing the CAP37 protein.

The CAP37-enriched fraction obtained from the molecular sieve column was further processed using hydrophobic high performance liquid chromatography (HPLC) (BIO-GEL TSK phenyl 5PW column, 7.5 mm x 0.75mm, packed with a 10 micron macroporous support having 1,000 angstrom pores and containing a low density of phenyl groups which promote strong but nondenaturing hydrophobic interactions with proteins, said column available from Biorad Laboratories). The proteins were eluted using a 60 min linear gradient from 1.7 M to O M $(NH_4)_2SO_4$, which contained 0.1 M sodium phosphate pH 7.0. The recovery was determined by the optical density of the proteins at 210 nm. The fractions obtained from the hydrophobic HPLC column were pooled independently and the $(NH4)_2SO_4$ removed by dialysis

employing a stirred cell concentrator (YM-5 membrane, Amicon) with 0.2 M sodium acetate buffer, pH= 4.0.

The purity of the fractions was determined by SDS-PAGE, western blot analysis and ELISA. All these results confirm that CAP37 prepared in this manner is devoid of other contaminating granule proteins which include the defensins, cathepsin G, myeloperoxidase, lactoferrin, and CAP57, and thus indicates that the outlined method yields a highly purified preparation of CAP37.

An important aspect of the production of CAP37 was to keep it free from endotoxin contamination. All reagents and buffers were prepared using pyrogen-free water and tested for endotoxin contamination using the Limulus amoebocyte assay (Whittaker Products). All glassware used was pyrogen-free. The starting material for each column was always checked for endotoxin contamination before it was applied to the column and, most importantly, the final product was always checked for the presence of endotoxin before use.

As seen in Fig. 12, the elution profile from the hydrophobic HPLC column indicated the presence of two proteins. CAP37 was confined to Peak 2, the more hydrophobic peak. Peak 1 was found to contain cathepsin G, as judged by SDS-PAGE.

SDS-PAGE was performed to depict the stages of purification of CAP37 from normal human crude granule extract (CGE). The analysis was performed according to the method described by Laemmli. Nature, 227: 680, 1970. The

samples were solubilized in 0.625M Tris (pH 6.8), 4% (w/v) SDS, and 1% (v/v) beta mercapto ethanol, at 100°C for 5 min and analyzed on a 12.5% gel essentially according to the method of Laemmli. Nature 227: 680, 1970. The analysis was performed under reducing conditions with a 12.5 % separating gel and a 4% stacking gel as described by Laemmli. Nature 227: 680-685, 1970. The ratio of the acrylamide to the bisacrylamide was 37.5. Electrophoresis was carried out until the bromophenol blue dye reached the bottom of the separating gel. The gel was removed and fixed in a 25% isopropanol, 7% acetic acid mixture. The gel was oxidized and stained with silver to visualize the protein bands.

The gel was silver stained according to well established protocols. Lane 1 contained 2 μg "rainbow" molecular weight markers (Amersham). Lane 2 contained 5 μg of crude granule extract (CGE). Lane 3 contained 400 ng of peak C material obtained from the G-75 column which served as the starting material for the HPLC column. Lane 4 contained 350 ng of peak 2 (CAP37) from the HPLC column. Lane 5 contained 350 ng of peak 1 from the HPLC column and lane 6 contained purified cathepsin G. The molecular weight standards used included myosin (M_r 200,000), phophorylase b (M_r 92,500), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,300).

The CAP37 material from peak 2 (lane 4) migrated in SDS-PAGE as three extremely closely spaced bands with a molecular weight near 37,000 daltons. This electrophoretic behavior probably indicates glycosylation of the protein, rather than any heterogeneity of the protein preparation. Furthermore, the fact that this preparation yielded an unambiguous amino acid sequence would also tend to indicate the absence of heterogeneity.

Judging from the nearly identical migration patterns of peak 1 from the HPLC Column (lane 6) and cathepsin G (lane 5), it may be concluded that this peak 1 material is cathepsin G. This was confirmed by the western blots.

The fractions obtained from the HPLC column were also analyzed by western blots. Western blot analysis was carried out according to the method of Towbin et al. (Proc. Natl. Acad. Sci. USA. 76: 4350-4354, 1979) with some modifications. Following electrophoresis on a 12.5 % SDS-PAGE gel, the proteins were transferred onto a nitrocellulose membrane (Biorad, Richmond, CA) in a TE series, Transphor Electrophoresis Unit (Hoefer Scientific Instruments, CA) under constant current (200 mA) at 11°C for 1.5 hr. The concentration of the methanol in the buffer was reduced from 20% to 5% (v/v) and the pH raised to 9. The protein was detected on the nitrocellulose membrane using a mouse antibody to CAP37 (1: 100 dilution) and an alkaline phosphatase immunoblotting system with the addition of 10 units per ml heparin sulfate buffer (Eastman, Kodak)

(Spitznagel et al., J. Immunology 139: 1291-1296, 1987) in the wash buffer (0.15 M NaCl, 0.01M Tris HCl (pH 7.5), 0.01% v/v TWEEN 20). Color development was obtained with the nitroblue tetrazolium/BCIP system.

The results of the western blot confirmed the SDS-PAGE results. Lane 1 contained molecular weight markers as described for the SDS-PAGE. Lanes 2 and 6 contained crude granule extract. Lanes 3 and 7 contained peak C material from the G-75 column. Lanes 4 and 8 contained peak 2 from the HPLC column and lanes 5 and 9 contained peak 1 from the HPLC column. The protein concentrations of all the samples loaded onto the gel were the same as described above.

Lanes 1 to 5 were probed with goat anti-human cathepsin G (1:2000 dilution) and lanes 6 to 9 were probed with mouse anti-human CAP37 (1: 100 dilution). The second antibody was conjugated to alkaline phosphatase (1: 7500 dilution, Promega). Color development was obtained using the nitroblue tetrazolium/ BCIP system as outlined in the manufacturers handbook (Promega). The goat anti-human cathepsin G antiserum reacted with crude granule extract, peak C material and peak 1 from the HPLC column. It did not react with CAP37 (peak 2 material from the HPLC column). On the other hand, the antiserum to CAP37 reacted with peak 2 material but not with the cathepsin G. This indicates that CAP37 was totally separated from the contaminating cathepsin G in the final step of the purification which is performed on the hydrophobic HPLC column.

The proteins obtained from the HPLC column were further analyzed by ELISA which confirmed the purity of the CAP37 preparation. The ELISA method used is described in Pereira, et al., J. of Immunological Methods 117: 115- 120, 1989, and has proven to be a reproducible and sensitive method for the detection of cationic proteins. Briefly, the protein was attached to a 96 well microtitre plate (NUNC Immunoplate I, VWR Scientific) which was pre-treated with poly-L-lysine. Following an overnight incubation at 4°C, the plate containing the antigen was washed four times in phosphate buffered saline (pH 7.4). Non-specific sites on the plate were then blocked with a heparin containing phosphate buffer, at room temperature for 1 hr (Pereira et al., 1989). The plates were washed and the primary antibody was then added to the plate and incubated for 1 hr at 37°C. Following this incubation the plate was washed and the second antibody was added and the plate incubated for 1 hr at 37°C. The second antibody was a goat anti-mouse immunoglobulin which is conjugated to horse-radish peroxidase. The color development step involves incubating the substrate (3.7 mM O-phenylenediamine) in citrate phosphate buffer, pH 5.0, containing 0.24 μl/ml 30% H_2O_2 at room temperature for 30 min. The reaction is stopped with 2.5 M H_2SO_4 and the absorbance read immediately using a TITERTEK multiscan plate reader (Flow Laboratories) at 492 nm.

The results of the ELISA confirmed the results of the other analyses. The same samples as used for the SDS-PAGE were also used in the ELISA. Primary antibodies were prepared against each of these samples. The material from Peak 2 reacted only with the antibodies prepared against a pure CAP37, while the material from Peak 1 reacted with the antibody prepared against Cathepsin G. The crude granule extract reacted with all the antibodies and the Peak C material reacted with antibodies for CAP37 and for Cathepsin G.

For amino acid sequencing of the CAP37 protein a final desalting step using reverse phase HPLC was employed. A DYNAMAX 300A C8 column was equilibrated with 0.1% Trifluoroacetic acid in water the above-purified material was then applied to the column. Elution of the purified protein was effected by a 30 minute elution with a 0-80% gradient of 0.1% Trifluoroacetic acid in acetonitrile at a flow rate of 1 ml per minute. The CAP37 protein containing sample corresponded to the 16.14 minute peak, as determined by SDS-PAGE: this fraction was concentrated in a Savant Instruments SPEED VAC.

C. Antibodies to Purified CAP37 Protein

Ascites fluid containing antibodies against CAP37 protein was prepared by injecting BALB/c mice (Jackson Laboratories) with 250 ng of alum-adsorbed (Herbert) CAP37 protein emulsified in an equal volume of Freund's complete

and Freund's incomplete adjuvant (Difco Laboratories). Mice were subcutaneously injected with a total volume of 200 µl of the above suspension. The mice were boosted three weeks later with 250 ng of alum-adsorbed CAP37 injected intraperitoneally. At the same time the mice were injected with 1 ml of pristane (2,6,10,14 - tetramethyl pentadecane, Sigma). One week later 10_7 SP2/0 mouse myeloma cells were injected intraperitoneally (Lacy et al.). Ascites fluid was collected. The specificity of the ascites fluid reactivity against the CAP37 protein was demonstrated in ELISA assays (Pereira et al.) using CAP37, CAP57 (a cationic antimicrobial protein of mol. wt. 57,000 Da; Spitznagel et al.), lactoferrin, myeloperoxidase, cathepsin G and lysozyme as the antigens. Further, no cross reactivity between Cathepsin G and CAP37 protein was observed by western blot analysis (Ausubel et al.).

For the immunocytochemical studies, the final cell concentration was adjusted to 1 x 10^6 nucleated cells per ml in 10% heat inactivated fetal bovine serum (Hyclone Laboratories Inc., Utah) in PBS. One hundred microlitres of the cell suspension was cytocentrifuged onto glass slides. The cells were fixed in buffered formol acetone, pH 7.2 at 4°C for 60 seconds. The staining was performed using the VECTASTAIN Avidin Biotin Complex - Glucose oxidase (ABC-GO) technique (Vectastain Laboratories, Burlingame, CA) as described previously (Spitznagel et al.). The above described monospecific polyvalent mouse anti CAP37 ascites

fluid (1:100) was used to stain the cells. Color development was obtained using the nitroblue tetrazolium salt (VECTASTAIN GO substrate kit I) at room temperature for 30 minutes. Normal mouse serum and phosphate buffered saline (PBS) served as the negative controls. Ascites fluid made against myeloperoxidase, a known marker of the primary granule of PMNs, served as the positive control.

D. Microsequencing of CAP37

The protein sequence analysis of CAP37 was performed using an Applied Biosystems Model 477A Protein/Peptide Sequencer with an on-line Applied Biosystems 120A PTH-Amino Acid Analyzer. Reagents and solvents were from Applied Biosystems, Foster City, CA. Phenylthiohydantoin (PTH)-derivatized amino acids formed sequentially by Edman degradation were separated using an Applied Biosystems PTH C-18 HPLC reverse phase microbore column (2.1 mm ID x 220 mm) by gradient elution. The sample was applied to an acid-etched glass-fiber filter which had been treated with 3 mg Biobrene (polybrene) and precycled. Peak identification and yield quantitation was based on a standard PTH-amino acid profile. The N-terminal end of the CAP37 protein was identified by standard microsequencing procedures (Applied Biosystems). Trypsin and chymotrypsin generated digestion fragments of CAP37, were separated and purified using hydrophobic and reverse-phase HPLC (Example 1-B). These fragments were then sequenced, as described above. When the

cloning of the cDNA was undertaken only the sequences shown in Table 1 had been determined. The sequences of the amino and carboxy terminii of the mature CAP37 protein are designated in Table 1.

Example 2

Chemotactic Properties of the Purified Human CAP37

A. In Vitro Chemotaxis Assays

Chemotaxis was measured using the modified Boyden chamber technique (Snyderman et al.). The leading front method (Zigmond et al.) was used to assess migration of monocytes and lymphocytes through a 8 μm filter (Millipore Corporation, Bedford, MA). PMN chemotaxis was measured using a 3 μm filter (Millipore). The purified CAP37 protein (Example 1) used in chemotaxis assays were endotoxin-free as determined by the Limulus Amoebocyte Lysate Assay (Whittaker Bioproducts, Inc., Walkersville, MD). The dilutions of CAP37 and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were made in Geys buffer (Gibco) containing 2% endotoxin-free bovine serum albumin (BSA). Geys buffer containing 2% BSA served as the negative control, and a 10^{-8} M solution of FMLP as the positive control.

The chambers were incubated in a humidified atmosphere (6.2% CO_2) for 2 hours when testing monocytes and lymphocytes, and for 30 minutes when testing PMNs. The filters were then removed and processed as previously described (Snyderman et al.). The cells were viewed using

oil immersion and the distance the cells had migrated into the filter was determined over five different fields on the same slide (Zigmond et al.). Triplicate assays were performed for each experimental point.

The results are presented in Fig. 1. The data clearly show the strong chemotactic properties of the CAP37 protein.

CAP37 did not have any effect on PMN and lymphocyte chemotaxis in the range employed in these experiments. In addition to the observed chemotactic effect on human monocytes, CAP37 at a higher concentration of 1000 ng/ml (2.7×10^{-8} M) was chemotactic for rabbit monocytes as well. The effect was selective for monocytes since rabbit PMN did not show a chemotactic response towards CAP37.

To distinguish directed cell movement (chemotaxis) through the filters of a Boyden chamber as opposed to accelerated random cell motion (chemokinesis), the chemokinetic activity of CAP37 was determined by the checkerboard assay of Zigmond et al. The checkerboard assay demonstrated that in addition to its chemotactic properties, CAP37 has some chemokinetic effect on monocytes.

B. <u>In Vivo Chemotactic Assay</u>

The purpose of these experiments is to document that CAP37 when injected <u>in vivo</u> into mice will result in the emigration of monocytes/macrophages.

Female BALB/c mice (6 - 8 weeks) (Jackson Laboratory, Bar Harbor, MI) were injected intraperitoneally (i.p.) with

100 ng CAP37 (purified according to the method of the invention) per mouse in 2 ml RPMI-1640 serum-free medium (Mediatech). A control group of mice were injected i.p. with 2 ml brewers thioglycollate (4% w/v) (Difco Laboratories), a known stimulator of inflammatory cells whose action is well documented. A second control group of mice were injected i.p. with 2ml of RPMI-1640 alone.

(RPMI-1640 serum free medium from Mediatech has the following composition: $CaNO_3 \cdot 4H_2O$:100.00mg/L; KCl:400.00mg/L; $MgSO_4 \cdot 7H_2O$:100.00mg/L; NaCl:6000.00mg/L; $NaHCO_3$:2000.00mg/L; $Na_2HPO_4 \cdot 7H_2O$:1512.00mg/L; D-Glucose:2000.00mg/L; Glutathione (reduced):1.00mg/L; Phenol red:5.00mg/L; L-Arginine(free base):200.00mg/L; L-Asparagine:50.00mg/L; L-Aspartic acid:20.00mg/L; L-Cystine:50.00mg/L; L-Glutamic acid:20.00mg/L; L-Glutamine:300.00mg/L; Glycine:10.00mg/L; L-Histidine (free base):15.00mg/L; L-Hydroxyproline:20.00mg/L; L-Isoleucine (Allo free):50.00mg/L; L-Leucine (Methionine free):50.00mg/L; L-Lysine HCl:40.00mg/L; L-Methionine:15.00mg/L; L-Phenylalanine:15.00mg/L; L-Proline (Hydroxy L-Proline free):20.00mg/L; L-Serine:30.00mg/L; L-Threonine (Allo free):20.00mg/L; L-Tryptophan:5.00mg/L; L-Tyrosine:20.00mg/L; L-Valine:20.00mg/L; Biotin:0.20mg/L; D-Ca pantothenate:0.25mg/L; Choline chloride:3.00mg/L;

Folic acid:1.00mg/L; i-Inositol:35.00mg/L; Nicotinamide:1.00mg/L; Para-aminobenzoic acid:1.00mg/L; Pyridoxine HCl:1.00mg/L; Riboflavin:0.20mg/L; Thiamine HCl:1.00mg/L; and Vitamin B_{12}:0.005mg/L.)

At 6, 24, 48 and 72 hours following these i.p. injections, four mice in each group were sacrificed by CO_2 anesthesia. Ten ml sterile medium was injected into the peritoneal cavity of each mouse and the abdominal area gently massaged to dislodge the exudate cells. The medium containing the exudate cells was then aspirated from the peritoneal cavity using a 19 gauge needle attached to a syringe. The total number of cells in the peritoneal exudate was determined by counting the cells on a COULTER COUNTER (Coulter Electronics, Hialeah, Florida: The operation of the COUTLER COUNTER is based on electrical conductivity difference between particles and common diluent. Particles act as insulators, diluents as good conductors. The particles, suspended in an electrolyte, are forced through a small aperture through which an electrical current path has been established. As each particle displaces electrolyte in the aperture, a pulse essentially proportional to the particle volume is produced. Thus a 3-dimensional particle volume response is the basis for all sizing, regardless of position or orientation of the particle in solution.) One hundred μl of the peritoneal exudate was cytocentrifuged onto a glass slide and the cells stained with Wright's stain. A differential count of the

cells was made by counting the number of monocytes, lymphocytes, neutrophils, basophils and eosinophils per hundred consecutive cells. These experiments were performed to determine the time course of migration of neutrophils, monocytes, lymphocytes and other cells into the peritoneal cavity, in response to an i.p. injection of CAP37.

Traditionally neutrophils are the first cells to migrate into the peritoneal cavity, and they do so about 6 hours after the i.p. injection. Monocytes emigrate much later, generally 72 hours to 6 days after the i.p. injection.

The results, shown in Table 1 below, indicated that an injection of 100 ng of CAP37 into the peritoneal cavity, moderately increased the overall numbers of cells emigrating into the peritoneal cavity. Nevertheless, monocytes appeared in the peritoneal cavity much earlier (24 hours) than when thioglycollate was used. Furthermore, the percentage of monocytes elicited with CAP37 (55%) was much greater than when sodium thioglycollate (33%) was used even at the 72 hour time point. A further interesting observation was the dramatic reduction in neutrophils by 24 hours with the CAP37 injection whereas, with thioglycollate, the neutrophils persist at a very high percentage up to 48 hours (36%), and are still present at 72 hours (21%).

These responses with CAP37 contrasted markedly with responses to endotoxin in which far greater cell numbers are found to endotoxin in which far greater cell numbers are

found and PMN persist as the predominant cell type for at least 48 hours (Snyderman et al, J. Expe. Med. 134: 1131, 1971). The migration of monocytes into the peritoneal cavity following CAP37 injection into the peritoneal cavity of mice demonstrates the potent monocyte chemoattractant capacity of CAP37 in this in vivo animal model.

Table 1: Migration of cells following intraperitoneal injection with the inflammatory stimulus indicated.

Inflammatory Stimulus[a]	Hours post Injection	Total Cells[b] x 106	%monocytes	%PMN
None	0	3.1	18	30
RPMI-1640	6	1.5	15	42
	24	2.2	12	11
	48	3.1	14	28
	72	2.8	43	5
Thioglycollate	6	9.5	4	65
	24	9.1	5	77
	48	8.4	20	36
	72	11.1	33	21
CAP 37	6	3.6	7	65
	24	3.8	27	26
	48	3.5	48	4
	72	3.3	55	3

[a] Four mice per group
[b] Mean value for each group of four mice

Example 3

Comparison of the CAP37 protein amino terminal sequence

Using the first 45 amino acids of the amino terminal end of the CAP37 protein, a search of a protein sequence data base was made using the **FASTAMAIL** program through the **BIONET** network. This search revealed substantial homologies with the amino termini of a subset of serine proteases which mediate a number of functions involved in the inflammation response (see Table 2: elastase, ELAST; complement factor D, FACTD; bovine plasminogen, PLASM; cathepsin G, CATG; rat mast cell protease I and II, RMCPI and RMCPII; cytotoxic T cell I, CCPI; cytotoxic T cell protease -- H Factor, HF).

The closest over-all homologies were obtained with two PMN-derived granular proteins: a homology of 57.5% with human elastase (Sinha et al.) also known as medullasin (Okano et al.) and a homology of 45% with human cathepsin G (Salvesen et al.). Other specific homologous sequences were seen with bovine plasminogen (45%) and human complement factor D (45.5%) (Johnson et al.; Nieman et al. et al.), which is the first enzyme involved in the activation of the alternative complement pathway.

Two other groups of proteins which demonstrated strong homology with the CAP37 protein were: (a) the group of serine protease-like molecules derived from granules of atypical rat mast cells -- rat mast cell protease I (Woodbury et al.), RMCPI, 38.6%, and rat mast cell protease II, RMCPII, 40% (Benfey et al.); and (b) two proteins from

cytotoxic T cells -- cytotoxic T cell protease I (Lobe et al.), CCPI, 40%, and cytotoxic T cell protease factor H (Gershenfeld et al.), HF, 38.6%.

Example 4

Isolation of a First cDNA having Homology to Human CAP37 Protein

A. Preparation of poly-A mRNA

The starting material for the isolation of human CAP37-encoding cDNA was tumor cell line HL60 (ATCC CCL240). Using the anti-CAP37 specific antibodies that were described above, the presence of an antigen reacting with these antibodies was shown to be present in the HL60 cell line: this cell line was chosen as the starting material for cloning a CAP37 encoding cDNA. All of the following procedures were carried out on ice and with RNase-free materials.

Monolayers of HL60 were grown to confluence using standard sera-supplemented minimal media (DMEM/10% Fetal Calf Serum, 50 units/ml penicillin and 50 μg/ml streptomycin) in 85 mm plates. Cells were grown to a density of 10^6 cells/ml. Each plate was placed on ice and washed four times with ice-cold phosphate buffered saline (PBS) and a final 2 ml aliquot of PBS added to each plate. The cells were harvested from each plate by scraping, transferred by pipette to COREX centrifuge tubes, and held

on ice. When all the plates were harvested the cells were pelleted by centrifugation at 2000 X g for 5 minutes at 4°C.

RNA was isolated from 10^8 cells by the previously described method of Chirgwin et al. using guanidine isothiocyanate.

B. Selecting the poly(A) enriched mRNA fraction.

Oligo(dT) cellulose (0.1-1.0 g; purchased from Pharmacia) was suspended in 1-5 ml of elution buffer (distilled H_2O, 1mM EDTA), poured into a 1- to 4-ml disposable column, and washed with 5 column volumes of binding buffer (0.01 M Tris-HCl at pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS). The isolated RNA was resuspended at 1-5 mg/ml in elution buffer, heated to 65°c for 5 min, quickly cooled in ice, diluted with an equal volume of 2X binding buffer, and applied to the column: the flow-through was collected and reapplied to the column. The column was washed with 5-10 volumes of binding buffer and 5 volumes of wash buffer (0.01 M Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA).

The bound RNA was eluted with 2-3 column volumes of elution buffer; subsequently, it is adjusted to a final concentration of 0.5 M NaCl (with 5 M NaCl or 2X binding buffer), re-bound, re-washed, and re-eluted as described above. The RNA in the final eluate was recovered by addition of 0.1 volumes of 3 M sodium acetate and precipitation with 2.5 volumes of ethanol. Sodium dodecyl

sulfate (SDS) is deliberately excluded from the wash and elution buffers to avoid precipitating the detergent from ethanol along with the RNA.

The final RNA sample was precipitated and dissolved in sterile water to a final RNA concentration of approximately 1μg/μl.

C. First-Strand cDNA Synthesis.

Double-stranded (ds) cDNA was synthesized using the Amersham cDNA synthesis kit. Briefly, 5 μg of mRNA was added to the following reaction mixture: first strand synthesis reaction buffer, sodium pyrophosphate solution, human placental ribonuclease inhibitor (HPRI, 50 units), deoxynucleotide triphosphate mix (1 mM dATP, 1 mM dGTP, 1 mM dTTP, and 0.5 mMdCTP), 4 μg oligo-dT, 100 units reverse transcriptase, and 5 μCi (α-^{32}P)-dCTP in a total volume of 50 μl. The reaction was incubated at 42°C for 60 minutes.

D. Second-Strand cDNA Synthesis.

A final volume (250μl) containing the first strand synthesis mix was mixed with E. coli ribonuclease (4 units) and E. coli DNA polymerase I (115 units). This mixture was incubated at 12°C for 60 minutes, 22°C for 60 minutes, and 70°C for 10 minutes. T_4 DNA polymerase (10 units) was added and the sample incubated at 37°C for 10 minutes. The ds cDNA was phenol/chloroform extracted, chloroform extracted, and ethanol precipitated (Maniatis et al.).

E. Cloning of the Double Stranded cDNA.

For ease of manipulation the double stranded cDNA was tailed with EcoRI linkers in the following manner.

The cDNA was then resuspended in water and treated with EcoRI methylase under the following conditions: 100 mM Tris HCl, pH=8.0; 10 mM EDTA; 80 μM S-adenosyl-methionine; 100 μg/ml bovine serum albumin; with approximately 100 units of EcoRI methylase (Promega) in a total volume of 50 μl incubated at 37°C for 60 minutes. The cDNA was then precipitated with ethanol.

The cDNA was resuspended in 5 ul of dH_2O to which was added 2 ul of 10x ligase buffer (0.5M Tris HCl, pH=7.4; 70mM $MgCl_2$; 10 and 1 ul t4 DNA ligase (100 units). The mixture was incubated at 16°C overnight. The reaction mixture was extracted once with phenol/chloroform and the cDNA precipitated with ethanol. The cDNA was then digested with EcoRI in a buffer containing 100 mM Tris-HCl (ph 7.5), 50 mM NaCl and EcoRI restriction enzyme (50 units) was incubated at 37°C for 3 hours. The ds cDNA was extracted, precipitated, resuspended, and separated from the EcoRI linkers using a Sephacryl S-400 column.

Finally, the cDNA was size fractionated as follows before insertion into the vector. The cDNA was resuspended in the following buffer: 0.6 M NaCl, 20 mM Tris HCl (pH=8.0), and 20 mM EDTA. This sample was then loaded on a SEPHACRYL S-400 column which was equilibrated with the same buffer. Fifty microliter aliquots were then collected and

assayed by electrophoresis through an alkaline agarose gel to identify the fractions containing labelled cDNA molecules of greater than 400 base pairs (bp) in length. These >400 bp fractions were then pooled and the cDNA precipitated with ethanol.

The vector for insertion of the >400 bp cDNA molecules was EcoRI digested and phosphatased gt10 (Stratagene). A Stratagene Gigapack Gold Packaging Extract Kit was used to package the Lambda gt10/cDNA ligation mixture. A primary titer of $1x10^7$ PFU's was obtained.

Plaques resulting from recombinant phage, carrying the cDNA, where then picked to fresh lawns of C600. Nitrocellulose filters (Schleicher and Schuell) were then presoaked in a cell suspension of C600Hfl (a less permissive host strain) and dried. The coated nitrocellulose filters were then layered onto the phage-containing agar plates to transfer phage particles to the filters. Duplicate filters were prepared.

When the bacterial lawn had grown and plaques were apparent the filters were then employed in in situ hybridization after the method of Woo. A primary screen of 500,000 clones at 50,000 PFU/plate was performed using duplicate filters. Briefly, the filters were lifted from the plates and placed on WHATMAN 3MM filters saturated with 0.5 M NaOH and 1.5 M NaCl to lyse the bacteria and phage particles. The filters were neutralized by successive transfer to WHATMAN filters saturated with, first, 0.5 M

Tris HCl, pH=7.4, with 1.5 M NaCl, and, second with 2X SSC (Maniatis et al.). The nitrocellulose filters were then blotted dry, air dried, and baked under vacuum at 68°C for 2 hours.

The filters were prehybridized with 1X PAM containing 30% formamide. (A 1 liter solution of 2X PAM contained: 200 ml 50X Denhardts; 500 ml 20X SSC; 100 ml 1M NaH2PO4, pH=7.0; 20 ml 100 mM PPI; 100 mM Na2HPO4; 100 ug/ml sonicated salmon sperm DNA; and, 120 mg ATP.) The prehybridization solution was removed. The hybridization was performed overnight, at 42°C, in a solution of 1 x PAM containing 30% formamide and a labeled probe set.

Two sets of probes were used to screen the duplicate filters. These probes were two sets of short oligonucleotides made to the amino-terminus of the CAP37 protein (Table 1) as follows:

Oligo 1 (Cap37a-1), non-coding strand, amino acids 18-24,

5'-C(GT)GCCTCC(TC)TG(AG)TT(CT)TG(AG)AT-3';

Oligo 2 (Cap37a-2), non-coding strand, amino acids 9-15,

5'-A(GA)GAAGGG(AG)AA(CT)TG(CATG)C(GT)GGG-3'.

Bases presented in parenthesis are alternative insertions at that site.

The two sets of probes were 5' end-labelled using [^{32}P]ATP and T_4 Polynucleotide kinase (Bethesda Research Laboratories, 5'DNA Terminus Labelling System). Each set of

duplicate filters were hybridized, as described above, with one of the probe sets. The filters were then washed as follows: 2 x SSC wash at 4°C (2x15 minutes) followed by a 3 M $(CH3)_4NCl$ wash at 53°C (2x15 minutes). The filters were then dried and subjected to radioautography at -20°C using intensifying screens. The duplicate filters showed one recombinant plaque that was convincingly positive with both probes.

Example 5

Analysis of the First cDNA insert in Lambda gt10

The restriction map of the cloned cDNA was determined; no internal EcoRI sites were detected in the sequence. The approximately 850 bp insert was removed from the gt10 vector by digestion with EcoRI; the corresponding DNA fragment was resolved and subsequently isolated from a polyacrylamide gel (Maniatis et al.). This EcoRI fragment was then cloned into the unique EcoRI site of the vector pcDNAI (Fig. 2; Invitrogen, San Diego, CA).

The vector pcDNAI has a Col E1 origin of replication derived from pBR322, the CMV promoter sequences and a polyadenylation signal available for expression in mammalian cells (such as COS7), and a poly-linker flanked by opposing T_7 and SP6 promoters providing for ease of expression of inserted DNA sequences in bacteria. The vector is also useful for DNA sequencing in that region of the poly-linker

provides for the use of a universal primer homologous to the T_7 and SP6 promoters.

The region containing the CAP37 coding region was sequenced by standard double-strand sequencing methods (Pharmacia Sequencing Kit, Pharmacia), starting with the universal sequence primer discussed above, as well as unique synthetic oligonucleotide primers derived from the CAP37 coding sequence as they were needed. The sequence of the first cDNA clone having homology to the CAP37 coding region is shown in Table 3.

Example 6

Characteristics of the First cDNA

The first cDNA sequence was translated into its corresponding amino acid (aa) sequence. A continuous open reading frame exits from nucleotide 176 to 856. This sequence was then aligned to the peptide fragments determined from microsequencing of the purified CAP37 protein (Table 1). A DNA sequence encoding a protein sequence corresponding to the amino terminus of the mature CAP37 protein sequence begins at nucleotide 320. The cDNA encodes a protein in which the first amino acid of the mature protein is preceded by an in-frame open reading frame corresponding to an additional 48 amino acids. Further, the carboxy terminus of the cDNA encoded protein contained an extension, Gly Pro Ala, not present in the mature protein.

In matching the translated cDNA to the protein sequences in Table 1 a problem was encountered in the cDNA sequence. The sequence of interest in CAP37 first cDNA is as follows:

```
ATG CTG CTT CAG AGG TTT GTC AAC GTG ACT GTG ACC CCC GAG GAC
 M   L   L   Q * R   F   V   N   V   T   V   T   P   E   D
CAG TGT CGC CCC ACC ACC GTG TGC ACC GGT GTG CTC ACC CGC CGC
 Q   C   R   P   N   N   V   C   T   G   V   L   T   R   R
GGT GGC ATC TCG AAT GGG GAC GGG GGC ACC CCC CTC GTC TGC GAG
 G   G   I   C   N   G   D   G   G   T   P   L   V   C   E
CCG CTG GCC CAC GGC
 G   L   A   H   G.
```

This sequence corresponds in part to PE-V10, an internal peptide of the CAP37 protein (amino and carboxy terminii were identified, see Table 1). A partial sequence of the PE-V10 peptide follows here:

Ala Gly Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro * Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln Cys Arg.

The 19 amino acids preceding the asterisk in the PE-V10 sequence were not represented in the first cDNA: the point of divergence in the cDNA sequence is also marked by an asterisk. Accordingly, while the first cDNA apparently coded for the majority of the CAP37 protein, it was not the correct cDNA.

The mature C-terminal sequence of the CAP37 protein (peptide PE-T19) indicates that there is also a carboxy

terminal extension present in the cDNA CAP37 protein coding sequence which is not present in the mature protein.

Example 7

Isolation of a Second cDNA having Homology to Human CAP37 Protein

A. Amplification of a fragment spanning the region of discrepancy between the cDNA sequence and the protein sequence.

As a first step to clone a cDNA containing the complete CAP37 coding sequence, an attempt was made to amplify a specific DNA fragment which would span the region where the discrepancy between the cDNA sequence and the protein sequence was observed. A degenerate oligonucleotide probe corresponding to the amino acids CQVAGWG (underlined in the CAP37 peptide sequence presented in Example 6) was synthesized. The oligonucleotide was a 20mer (256 fold degenerate, designated Intr1c), synthesized as follows:

5'TG(CT)CA(AG)GT(ACTG)GC(ACTG)GG(ACTG)TGGGG 3',

where the bases in parenthesis are alternative substitutions at that site.

As a second primer for use in DNA amplification a number of 20mer primers were designed from the sequence of the non-coding strand of CAP37 first cDNA.

Total human genomic DNA was used as template in DNA amplification reactions (Mullis; Scharf et al.) involving

the above described primers. The amplification reactions were divided into two samples and each set of samples were fractionated on an agarose gel. The samples in one half of the gel (representing a complete set of amplification reactions) were transferred to nitrocellulose (Maniatis et al.). The nitrocellulose filter was hybridized (Maniatis et al.) with radiolabelled CAP37 first cDNA (Bethesda Research Laboratories Nick Translation Kit) as a probe.

The Southern blot hybridization showed one positive band. This band was generated using primer Intr1c (above) and a non-coding strand 20 mer designated 669nc (corresponding to base pairs 652-671 of CAP37 first cDNA, Table 3). The region of the second half of the agarose gel corresponding to the positive band was excised and the DNA extracted, phenol/chloroform extracted, and precipitated (Ausubel et al.). The band was then sequenced using the Intr1c/669nc primers (Pharmacia Sequencing Kit, Pharmacia).

The sequence of Intr1c/669nc generated band was as follows:

TGTAG GTT GCG GGT TGG GGG AGC CAG CAC AGT GGG GGG CGT CTC TCC CGT

V A G W G S Q H S G G R L S R

TTT CCC AGG TTC GTC AAC GTG ACT GTG ACC CCC GAG GAC CAG TGT CGC CCC

60

F P R F V N V T V T P E D Q C R P

AAC AAC GTG TGC ACC GGT GTG CTC

N N V C T G V L .

B. Second Screening of the lambda gt10/HL-60 cDNA library for a CAP37 cDNA.

A 50mer corresponding to a region of the Intrlc/669nc sequence which was not present in the CAP37 first cDNA clone was synthesized and used as a probe to screen 200,000 clones at 50,000 PFU/plate (as in Example 4).

The sequence of the oligonucleotide probe was as follows:

5' TGTCAGGTTGCGGGTTGGGGGAGCCAGCACAGTGGGGGGCGTCTCTCCCG 3'.

On an initial screen 11 positives were identified of which 5 remained positive following a secondary screen. The EcoRI cDNA fragment of one of these clones, designated 6a.1, was subcloned into the pcDNAI vector and sequenced using dideoxy sequencing: the 6a.1 insert subclone in pcDNAI was designated pcDNA-CAP#2. The complete DNA sequence of the 6a.1 clone is illustrated in Table 4. The plasmid pcDNA-CAP#2 has been deposited under the terms of the Budapest Treaty with the American Type Culture Collection (12301 Parklawn Dr., Rockville MD 20852 USA) and given accession number ATCC 68340.

Example 8

Characteristics of the 6a.1 cDNA Encoded Protein

The 6a.1 cDNA sequence was translated into its corresponding amino acid (aa) sequence. A continuous open reading frame exists from nucleotide 4 to 756. This sequence was then aligned to the peptide fragment sequences obtained from microsequencing of the purified CAP37 protein (Example 1). The corresponding DNA coding sequence begins at nucleotide 82. All of the protein sequences determined by microsequencing of the purified CAP37 protein had corresponding protein sequences in the 6a.1 cDNA encoded protein.

The 6a.1 cDNA encodes a protein in which the first amino acid of the coding sequence of the mature protein is preceded by an in-frame open reading frame corresponding to an additional 26 amino acids: these 26 amino acids do not correspond to those seen in the first cDNA protein. The first approximately 20 amino acids of this region have a high degree of hydrophobicity (Fig. 3). The length of the sequence and the degree of hydrophobicity make this sequence an ideal candidate for a eucaryotic secretory signal sequence. The hydropathy index plot was generated using the SOAP program of PCGENE (Intelligenetics, Mountain View, CA). The SOAP program uses the method of Kyte et al to plot the hydropathicity of the protein along its sequence. The interval used for the computation was 11 amino acids.

The cDNA encoded protein apparently has a carboxy terminal extension relative to the mature CAP37 protein, i.e. Gly Pro Ala.

The portion of the open reading frame corresponding to the mature form of the protein has an estimated molecular weight of 24,276 daltons. The molecular weight previously determined for the CAP37 protein, i.e. approximately 37,000 daltons (Shafer et al., 1986), was obtained from SDS-PAGE of the purified protein. The discrepancy in molecular weight is most likely the result of post-translational modification of the CAP37 protein. For example, the CAP37 sequence has three potential N-glycosylation sites, as determined by PROSITE analysis (PCGENE Intelligenetics, Mountain View, CA) of the 6a.1 cDNA coding sequence. These potential glycosylation sites are located at asparagine residues 100, 114, and 145 of the mature sequence.

Using the method of Hopp et al. (ANTIGEN program, PCGENE Intelligenetics, Mountain View, CA) the primary antigenic determinants of the CAP37 protein sequence were determined. The three highest points of hydrophilicity are from residues 61 to 66, 5 to 10, and 150 to 156 of the mature protein sequence, with the highest average hydrophlicity value being for residues 61-66.

Example 9

Expression of Recombinant CAP37 Protein from the 6a.1 cDNA Coding Sequence

A. Expression of CAP37 in E. coli.

The following primers are used for DNA amplification of the CAP37 coding DNA fragment from the CAP37 cDNA clone (Example 7): 5'-ATCGTTGGCGGCCGGAAGGCG-3'; and, 5'-TTGGGCCCTGGCCCCGGTCGG-3'. These primers result in the amplification of the CAP37 mature protein coding sequence. If a 5' methionine is required for expression the first primer can be modified to 5'-ATGATCGTTGGCGGCCGGAAGGCG-3' to generate amplified fragments encoding a 5' methionine. Primers can also be designed to result in amplification of the precursor form of the CAP37 protein, for example, 5'-ATGGGGGAGGGTGGGTCC-3', and the same second primer as above.

Following confirmation by DNA sequencing, the amplified DNA fragment is blunt-end ligated (Maniatis et al.) in-frame into a plasmid expression vector (Smith et al.) designed to direct synthesis of foreign polypeptides as fusions with the protein Sj26; Sj26 is a glutathione S-transferase protein from Schistosoma japonicum (Smith et al.). Further, Smith et al. have described an expression vector encoding a thrombin cleavage site immediately adjacent the carboxy terminus of Sj26 at the site where the amino-terminal of the foreign protein is fused. E. coli strain JM101 is then transformed (Maniatis et al.) with either (i) the plasmid

containing the fusion protein coding sequences, or (ii) the parent plasmid without the CAP37 sequences.

The transformed cells are grown, expression of the desired proteins induced with IPTG (Smith et al.), and the cells grown a further 3-6 hours before harvest. As a control transformed cells are also grown and not induced with IPTG.

The harvested cells are then lysed (Smith et al.). A portion of each lysate is subjected to SDS-PAGE to test for expression of the fusion protein by size comparison to the native Sj26 protein and to protein molecular weight size standards. A band traveling at the molecular weight predicted for the fusion protein is expected from the lysate containing the fusion protein coding sequences, but not from lysates from cells transformed with parent vector alone or from those not induced with IPTG.

The bulk of the fusion-protein-containing lysate is treated with immobilized glutathione to purify the fusion protein (Smith et al.). This purified fusion protein is then subjected to SDS-PAGE to examine the relative purity of the preparation. The purified fusion protein is cleaved with thrombin (Boehringer-Mannheim) and the CAP37 separated from the Sj26 protein by chromatography (e.g., size exclusion chromatography followed by an ion exchange column as in Example 1).

B. Expression of CAP37 in Yeast.

For expression of CAP37 in yeast, pre-pro-alpha-factor/CAP37 gene fusions are constructed. The DNA amplified CAP37 cDNA used above for construction of E. coli vectors is also used for construction of yeast vectors. The expression vector contains the yeast alcohol dehydrogenase I transcription promoter and terminator (Oeda et al.). The CAP37 cDNA is blunt-end ligated, in-frame (Maniatis et al.) directly following the Lys-Arg dipeptide sequence of the pre-pro-alpha-factor leader (Kurjan et al.). This expression vector construct directs synthesis, secretion and processing of the hybrid protein in yeast.

In order to maximize protein product yield the expression vector containing the CAP37 cDNA is transformed (Ausubel et al.) into a yeast strain carrying the pep4 mutation (Jones); such strains are defective in four major vacuolar proteases and RNase. The CAP37 protein is purified from the media by a combination of size fractionation followed by ion-exchange chromatography (Example 1). Alternatively, the yeast cells are mechanically lysed (Guarente) and the CAP37 protein isolated from the lysate in the same manner.

C. Expression of CAP37 in Mammalian Cells.

(i) Modification of CAP37 cDNA.

The cDNA clone encoding CAP37 was modified to be bounded by a HindIII restriction site at the 5' end of the

sequence and a XbaI site at the 3' end as follows. Two primers for use in DNA amplification reactions were designed to be complementary to the ends of the CAP37 cDNA and contain the desired restriction sites. The sequence of the two primers used to prepare the DNA amplification product were:

5': 5'-CCGGAATTCCAAGCTTCCACCATGACCCGGCTGACAGTCCTGG-3'
HindIII

3': 5'-CCGGATCCTCTAGACCCTAGGCTGGCCCCGGTCCCGG-3'.
XbaI

The DNA was amplified as per the manufacturers instructions (Cetus-Perkin Elmer, Norwalk CT). The resulting 5' end contained a HindIII site followed by the consensus mammalian initiator sequence CCACCATG.

(ii) Construction of Vectors.

The above modified CAP37 cDNA was inserted into a series of expression vectors having the following properties:

1. Unique HindIII and XbaI cloning sites situated between a strong viral promoter (either CMV, SV40, or RSV) and a polyadenylation signal derived from SV40).

2. A selectable marker linked to the vector conferring either Genetimicin resistance or methotrexate resistance.

The vectors are shown in Fig. 4.

In Fig. 4, plasmid vector sequences derived from pUC18 are denoted by the thin line. The promoter preceding the CAP37 cDNA insert was joined to the pUC polylinker at the EcoRI site. The cytomegalovirus (CMV) promoter cassette (pCXSD-CAP37 and pCXRD-CAP37) was a 637 bp fragment which contains the promoter and enhancer of the immediate early gene 1 (IE1) of CMV (Seed). The promoter cassette was modified to be bounded by an EcoRI restriction site at the 5' end of the cassette and HindIII and BglII sites at the 3' end of the cassette. The SV40 sequences flanking the 3' end

of the CAP37 cDNA insert was composed of two elements from the SV40 early region. A 620 bp Sau3A fragment spanning the T antigen splice signal was joined to the 350 bp BclI-BamHI fragment of SV40 which spanned the polyadenylation signal of the SV40 early gene (Seed). The dihydrofolate reductase (DHFR) cassette was derived from a murine cDNA encoding DHFR and modified to contain a BglII site preceding the translational start site of the clone and ending at a SalI restriction site situated adjacent to the BglII restriction site of DHFR (Simonsen et al.). The SV40 E promoter cassette, at the 5' end of the DHFR insert in pCXSD-CAP37, was a 540 bp fragment which spanned the SV40 origin of replication, early, and late promoters, and is bounded by an EcoRI (Simonsen et al.). This SV40 promoter cassette can also be used for expression of the CAP37 cDNA (e.g., pSXRD-CAP37). The RSV promoter cassette, at the 5' end of DHFR in pCXRD-CAP37 and pSXRD-CAP37, was derived from the Rous Sarcoma Virus and spans the LTR, ending at a point 90 base pairs downstream of the transcriptional start site (Seed). The HBV 3' end of the each vector was a 585 bp BamHI-BglII fragment which spanned the HBV surface antigen polyadenylation signal (Simonsen et al.).

(iii) Derivation of Transformed Cell Lines.

Host cell lines which can be transformed using the above described vectors include human, mouse, and hamster. The preferred host cell line is a Chinese Hamster Ovary cell line (CHO) which has been rendered incapable of producing the enzyme dihydrofolate reductase (Urlaub et al.): the expression of CAP37 is not limited to this line. Typically, use of vectors containing a gene encoding dihydrofolate reductase (DHFR) has allowed for the efficient selection of stable cell lines expressing the transfected plasmid. In addition, such cell lines are capable of being selected with the folate analogue methotrexate, resulting in the emergence of cells having amplified copies of the DHFR cDNA and the

co-transfected sequences (Simonsen et al.; McIvor et al.; McGrogan et al., 1988 A).

Plasmid DNA containing the CAP37 protein encoding cDNA and the DHFR-encoding gene was transfected into CHO cells using the cationic liposome method (DOTMA). 2 x 10^5 cells were passaged into 5-25 flasks 48 hours prior to the transfection and allowed to grow in non-selective media. Ten ug of plasmid DNA was diluted to 50 ul with sterile distilled water and then added to 25 ug of DOTMA (Lipofectin, Bethesda Research labs) in a volume of 50 ul. The DNA was mixed and allowed to stand 30 minutes at room temperature. Immediately prior to the addition of the DNA to the cells, the T-flasks were washed three times with serum-free media. Three milliliters of serum-free media was added to each T-flask, after which the DOTMA-DNA mixture was added to the cells. The flasks were swirled and returned to the incubator for 4 hours. Three milliliters of fresh media with 20% serum was then added to each flask of cells and the cells allowed to incubate for 48-72 hours at which time the cells were removed by trypsinization, split into 10 cm dishes, and suspended in selective media containing methotrexate.

The cells were returned to the incubator and allowed to grow with twice-weekly changes of media. After 2-3 weeks, colonies become apparent. Cloning cylinders were used to isolate and subclone colonies arising from an individual transfected cell.

Both bulk populations and subclones were expanded for protein assay by Western blot assays (Ausubel et al.). Cell lines expressing CAP37 antigen were selected for amplified CAP37 expression by growing the cells in stepwise increasing levels of methotrexate (MTX). Cell lines resistant to 1 μM MTX were subcloned and characterized by Northern and Southern analysis (Maniatis et al.), doubling time, expression levels and stability of expression.

In addition to the above bacterial, yeast, and mammalian systems there are numerous expression systems, well known to one of ordinary skill in the art, available suited to the expression of CAP37 (eg., Kingsman et al.; Brake, 1989; Revel et al.; Moriarty et al.; Kopchick et al.).

For all of the above expression systems antibodies generated to the CAP37 protein, or portions thereof, (Examples 1 and 13) can be used to follow and to quantitate the CAP37 produced; the antibodies are particularly useful for following the purification of CAP37 as, for example, described above in the E. coli system.

Example 10

Preparing Cloned Peptide Fragments

A. DNA Fragment Digestion.

The amplified 6a.1 CAP37 cDNA described above in Example 9 is modified by treatment with EcoRI methylase under standard conditions (Promega). The DNA is phenol/chloroform extracted and ethanol precipitated. The modified DNA is suspended in a standard digest buffer (0.5M Tris HCl, pH 7.5; 1 mg/ml BSA; 10mM MnC12) to a concentration of about 1 mg/ml and digested with DNAse I at room temperature for various times (1-5 minutes). These reaction conditions were determined from a prior calibration study, in which the incubation time required to produce predominantly 100-200 basepair fragments was determined. The material was extracted with phenol/chloroform before ethanol precipitation.

The fragments in the digest mixture were blunt-ended using DNA Polymerase I (Maniatis et al.) and ligated with the EcoRI linkers. The fragments were then digested with EcoRI under standard conditions (Promega). The resultant fragments were analyzed by electrophoresis (5-10V/cm) on 1.2% agarose gels, using PhiX174/HaeIII and lambda/HindIII size markers. The 50-200 bp fraction was eluted onto NA45

strips (Schleicher and Schuell), which were then placed into 1.5 ml microtubes with eluting solution (1 M NaCl, 50 mM arginine, pH 9.0), and incubated at 67°C for 30-60 minutes. The eluted DNA was phenol/chloroform extracted and then precipitated with two volumes of ethanol. The pellet was resuspended in 20 µl TE buffer (0.01 M Tris HCl, pH 7.5, 0.001 M EDTA).

B. Cloning the Digest Fragments.

The pUC7 plasmid (Messing) is modified to encode a collagenase (EC 3.4.24.3, from Clostridium histolyticum, available from Boehringer Mannheim) cleavage site using the following linker:

GAT CCG CCT GCA GGC CCT GTA AGC TTG AGG
GC GGA CGT CCG GGA CAT TCG AAC TCC AGC T

BamHI HindIII SalI.

Insertion of the above linker between the BamHI and SalI sites of the pUC7 plasmid results in the introduction of a collagenase cleavage site (Pro Ala ¦ Gly Pro Val), and a diagnostic HindIII site into the plasmid (designated pUC7-C). Further, the linker maintains a contiguous open reading frame into the ß-galactosidase protein coding sequence. The phosphorylated oligonucleotides are synthesized by standard methods. The oligonucleotides are then mixed in an equimolar amount, heated and renatured by cooling to form the above duplex linker.

The pUC7-C plasmid is digested with EcoRI and treated with calf-intestinal phosphatase (Promega). The digest fragments from Part A were introduced into the EcoRI site by mixing 0.5-1.0 µg EcoRI-cleaved pUC7-C, 0.3-3 µl of the above sized fragments, 0.5 µl 10X ligation buffer (Maniatis et al.), 0.5 µl DNA ligase (200 units), and distilled water to 5 µl. The mixture was incubated overnight at 14°C.

The ligation mixtures are then transformed into E. coli strain JM101 and plated on YT media (Gibco) containing ampicillin (100 μg/ml) at a density of 300-500 colonies per plate. The plates are replica-plated to X-gal indicator plates (Miller), YT+amp, and to nitrocellulose filters (Grunstein et al.). Blue colonies are scored on the X-gal indicator plates and indicate possible in-frame fusions of an inserted DNA fragment (Part A) to the lacZ gene contained in the vector. The nitrocellulose filters are screened as follows. The amplified 6a.1 cDNA is digested with the restriction enzyme HaeIII under standard reaction conditions (New England Biolabs): the 6a.1 cDNA has 19 HaeIII sites. The resulting DNA fragments are then phenol/chloroform extracted and precipitated with ethanol. The DNA is resuspended and radioactively labelled by nick-translation (Bethesda Research Laboratories). The nitrocellulose filters are hybridized with the labelled 6a.1 cDNA as previously described (Grunstein et al.). The filters are then washed and subjected to autoradiography.

pUC7-C vectors which contain inserts from the 6a.1 cDNA will test positive by this screen. These positive colonies are further screened by comparison to the colonies on X-gal plates. Bacterial colonies which test positive for the presence of a fragment of the 6a.1 cDNA and are blue are candidates for in-frame fusions of fragments of the CAP37 coding sequence to β-galactosidase.

These candidates are picked to master plates. The candidates are inoculated into YT+ampicillin (Maniatis et al.) and grown to mid-log phase. The cultures are combined in groups of 5. The combined cells are pelleted by centrifugation and suspended in lysis buffer (10 mM Tris, pH=7.4, containing 2% Triton X-100™, with 1% aprotinin added just before use). The resuspended cells are frozen in liquid nitrogen, then thawed, resulting in substantially complete cell lysis. The lysate is treated with DNaseI to digest bacterial DNA, as evidence by a gradual loss of

viscosity in the lysate. Non-solubilized material is removed by centrifugation.

SEPAHAROSE 4B beads conjugated with anti-beta galactosidase are purchased from Promega. The beads are packed in a column and washed successively with phosphate-buffered saline with 0.02% sodium azide and 10 ml TX buffer (10 mM Tris buffer, pH 7.4, 1% aprotinin). The clarified lysate material is loaded on the SEPHAROSE column, the ends of the column are closed, and the column is placed on a rotary shaker for 2 hrs. at room temperature and 16 hours at 4°C. After the column settles, it is washed with TX buffer. The CAP37 protein fragments are released by collagenase cleavage (Scholtissek et al.; Ullmann). The eluent is then concentrated by ultrafiltration.

Example 11

Characterization of CAP37 Protein Fragments

A. Identification of CAP37 fragments having chemotactic properties.

The eluents generated in Example 10 are evaluated for their effects on human monocyte chemotaxis by the method of Cates et al. Briefly, one filter at a time is placed in the Boyden chamber (Neuroprobe, Bethesda MD). The eluent to be analyzed for its chemotactic activity is placed in the lower chamber. A cell suspension of monocytes is placed in the upper compartment. The filled chambers are then briefly spun in a Sorvall centrifuge, covered to prevent evaporation, and incubated at 37°C for approximately 120 minutes.

The filters are then removed from the Boyden chamber, stained, and fixed. The number of monocytes adhering to the

chemoattractant side of the filter represent a fraction of the cells that have migrated through the filter in response to the chemoattractant. The number of monocytes in a 5 X 5 mm grid is counted for 10 randomly selected high-power fields (10 X ocular, 45 X objective) for each filter and an average cell number per grid value determined.

The number of cells migrating in response to buffered HBSS (Hank's Balanced Salt Solution; Gibco, Grand Island NY) alone is also determined and is subtracted from the average number of cells per grid migrating in response to the potential chemoattractant. The number of cells migrating in response to the CAP37 protein, also determined as just described, is used as a positive control to represent 100% chemotactic activity. The eluents are evaluated as the percent of the control chemotactic activity as follows:

$$100 \text{ X } \frac{\text{corrected average number of cells per grid eluent}}{\text{corrected average number of cells per grid CAP37}}$$

An average of three filters per eluent are tested in addition to the above controls. When a pool of eluent is identified as containing a chemotactic agent, the clones comprising the pool are individually tested in the above manner to identify the clone(s) responsible for the chemoattractant property.

B. Identification of CAP37 fragments having lipopolysaccharide binding properties.

Pooled eluent is prepared as described in Example 10-A. The eluents are tested for lipopolysaccharide neutralizing capacity using the Limulus Amebocyte Lysate (LAL) Assay as previously described by Warren et al. Briefly, the pooled

eluent sample is diluted 1:1 in normal saline (USP Abbott Laboratories, Chicago IL) and added to serial two-fold dilutions, made in normal saline, of E. coli lipopolysaccharide (Whittaker Bioproducts, Inc., Walkersville, MD) contained in wells of a 96 well microtiter plate (Flow Laboratories, McLean VA). The mixtures are incubated for approximately 3 hours at 37°C followed by the addition of reconstituted LAL (Whittaker Bioproducts, Inc., Walkersville, MD). The reactions are incubated for 1 hour at 37°C and then read using a microplate reader (Flow Laboratories, McLean VA) set at 380 nm.

To evaluate LPS neutralization capacity of the eluent, the results of the above assays are plotted and the LPS concentration needed to produce 50% of the maximal increase in $O.D._{380}$, i.e. the maximum O.D. produced in the presence of excess LPS, is determined by interpolation. This value is called the 50% limulus gelatin response (LR_{50}; Warren et al.).

Each microtiter plate has the following two controls: (i) a saline control, that is, no potential neutralizing agent; and, (ii) a dilution of purified CAP37 protein as a neutralizing agent control. LR_{50} values elevated above the saline control indicate potential LPS neutralizing peptide fragments of the CAP37 protein. When a pool of eluent is identified as containing a potential LPS neutralizing agent, the clones comprising the pool are individually tested in

the above manner to identify the clone(s) responsible for the LPS neutralizing property.

C. Characterization of Identified Clones.

The CAP37-encoding inserts of clones identified as of interest for either chemotactic or LPS binding properties are sequenced using dideoxynucleotide double-strand sequencing (Pharmacia Sequencing Kit, Pharmacia). Universal primers, corresponding to vector sequences which flank the inserts, are employed in the sequencing reactions. The insert sequences are then aligned with the complete CAP37 sequence to identify the locations of these regions of interest in the corresponding CAP37 protein sequence.

Example 12

Alternative Method for Identification of Peptide Fragments and Chemotactic Activity of said Fragments

The amino acid sequence of CAP37 was compared to that of elastase and cathepsin G, two serine proteases with which CAP37 has close homology. Because neither elastase nor cathepsin G exhibit monocyte chemotaxis, stretches of the CAP37 molecule which were the least homologous to either cathepsin G or elastase were selected as fragment likely to have monocyte chemotactic activity. Three such fragments, depicted in Table 5, were identified and synthesized using standard peptide synthesis techniques.

The three peptides were tested for chemotactic activity towards monocytes using the modified Boyden Chamber technique described in Example 2. The monocyte chemotactic activity of each peptide fragment at concentrations ranging from 10^{-7} to 10^{-13}M was compared with Geys buffer (negative

control), FMLP 10^{-8}M (positive control) and purified CAP37 10^{-9}M (positive control). Peptides 1 and 3 showed chemotactic activity for monocytes while peptide 2 did not. The effect of peptide 1 on monocyte chemotaxis is illustrated in Table 6 while the effect of peptide 3 on monocyte chemotaxis is illustrated in Table 7. The results with both peptides 1 and 3 show that significant chemotaxis occurs at 10^{-10}M concentration.

Example 13

Preparation of Anti-CAP37 Antibodies

The fusion protein containing the full length CAP37 protein and glutathione S-transferase (Example 9-A) is isolated from lysed bacteria. Transformed bacteria are streaked for single colonies, grown at 37°C overnight or until colonies are apparent. Individual colonies are inoculated into 1 ml of selection media and grown overnight. Saturated overnight bacterial culture are used to inoculate cultures, which are incubated with aeration to an O.D. of about 0.5-0.6. The cells are pelleted by centrifugation, and resuspended in lysis buffer (62 mM Tris, pH 7.5 containing 5% mercaptoethanol, 2.4 % SDS and 10% glycerol). The lysate is treated with DNaseI to digest bacterial DNA, as evidence by a gradual loss of viscosity in the lysate. Non-solubilized material was removed by centrifugation. The fusion protein is isolated from the bacterial lysate by treatment with immobilized-glutathione (Smith et al.).

For the isolation of CAP37/ß-galactosidase fusion proteins (Examples 10 and 11), the cells are pelleted by centrifugation and suspended in lysis buffer (10 mM Tris, pH 7.4 containing 2% TRITON X-100 and 1% aprotinin added just before use). The resuspended cells are frozen in liquid nitrogen, then thawed, resulting in substantially complete cell lysis. The lysates are treated with DNaseI to digest bacterial DNA, as evidence by a gradual loss of viscosity in

the lysate. Non-solubilized material is removed by centrifugation.

SEPHAROSE 4B beads conjugated with anti-beta galactosidase are purchased from Promega. The beads are packed in columns and washed successively with phosphate-buffered saline with 0.02% sodium azide and TX buffer (10 mM Tris buffer, pH 7.4, 1% aprotinin). The clarified lysates are loaded on the SEPHAROSE columns, the ends of the columns are closed, and the columns placed on a rotary shaker for 2 hours, at room temperature and 16 hours at 4°C. After the columns settle, they are washed with TX buffer. The fused proteins are eluted with 0.1 M carbonate/bicarbonate buffer, pH10. The eluate from each affinity column is concentrated by ultrafiltration using, for example, CENTRICON-30 cartridges. The final protein concentrate is resuspended in PBS buffer.

The individually purified fusion proteins are injected subcutaneously in Freund's adjuvant in rabbits. 1 mg of fused protein is injected at days 0 and 21, and rabbit serum is collected on days 42 and 56.

Control rabbits are similarly immunized with purified glutathione S-transferase (Sj26) protein obtained from control bacterial lysate and purified β-galactosidase obtained from commercial sources.

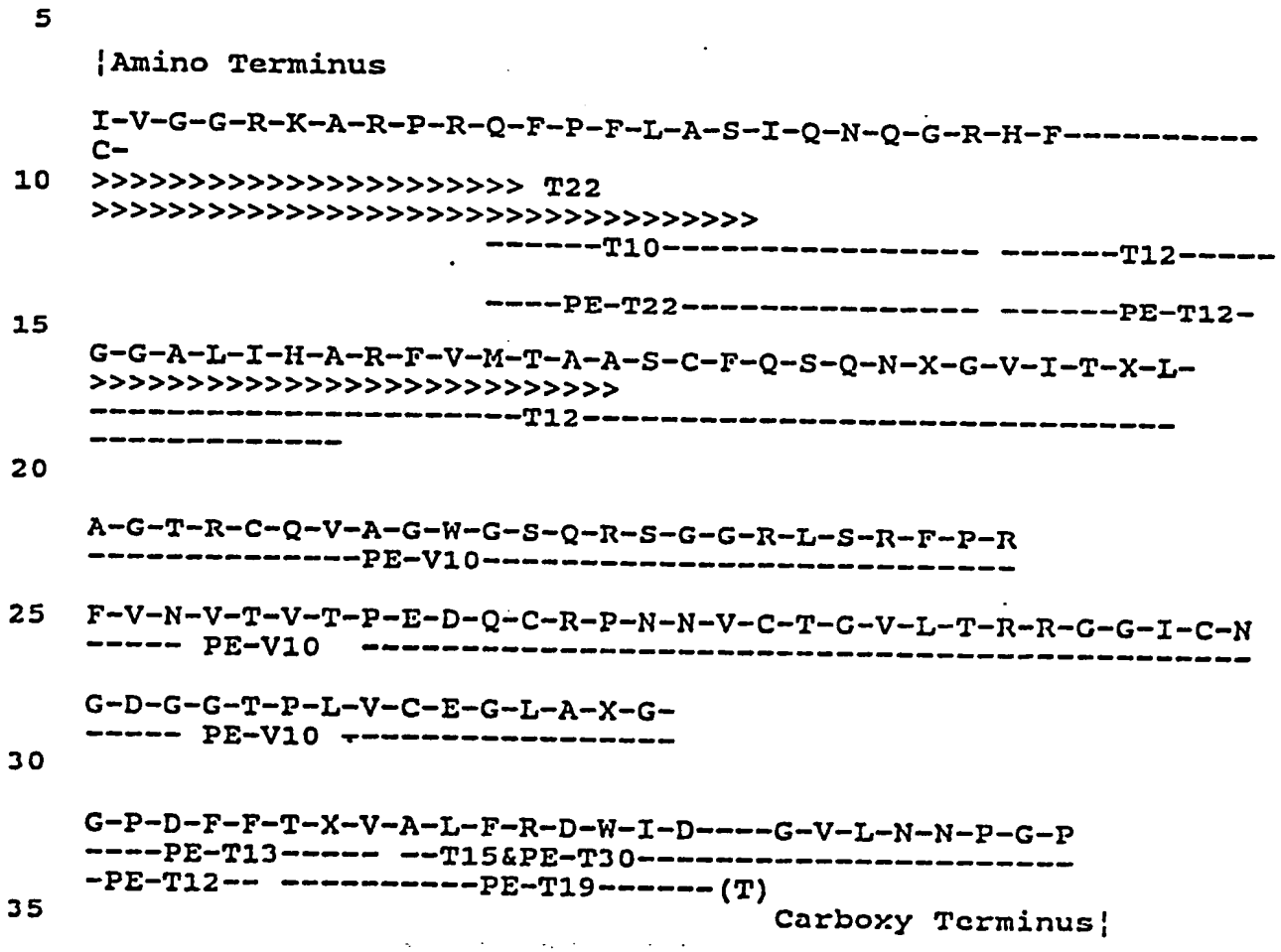
Minilysates are prepared (essentially as described above for the fusion proteins but omitting the affinity purification steps) from the following transformed bacterial cultures: (1) cells transformed with the CAP37/glutathione S-transferase fusion-encoding plasmid; (2) cells transformed with the glutathione S-transferase encoding plasmid; (3) cells transformed with each of the CAP37 peptide/β-galactosidase fusion-encoding plasmids (Example 11); and, (4) cells transformed with the β-galactosidase fusion-encoding plasmid. The minilysates and purified CAP37 protein were fractionated by SDS-PAGE, and the bands

transferred to nitrocellulose filters for Western blotting (Ausubel et al.).

Serum from control (Sj26) rabbits is expected to be immunoreactive with each of the Sj26 and Sj26 fused protein antigens. Serum from the animal immunized with CAP37/Sj26 fused protein is expected to be reactive with Sj26, Sj26 fused protein antigen, and purified CAP37 indicating some specific immunoreaction with CAP37. The serum from the animal immunized with CAP37/Sj26 fused protein may also immunoreact with the CAP37 peptide/β-galactosidase antigens, but is not expected to react with β-galactosidase antigen alone.

Serum from control (β-galactosidase) rabbits is expected to be immunoreactive with each of the β-galactosidase and β-galactosidase fused protein antigens. Serum from the animal immunized with CAP37/β-galactosidase fused protein is expected to be reactive with β-galactosidase, β-galactosidase-fused protein antigen, and purified CAP37 indicating some specific immunoreaction with CAP37. The serum from the animal immunized with CAP37/β-galactosidase fused protein may also immunoreact with the CAP37 peptide/Sj26 antigens, but is not expected to react with Sj26 antigen alone.

Although the invention has been described with reference to specific methods and compositions, it will be apparent to one skilled in the art how various modifications and applications of the methods may be made without departing from the invention.


¦Amino Terminus
I-V-G-G-R-K-A-R-P-R-Q-F-P-F-L-A-S-I-Q-N-Q-G-R-H-F----------
C-
>>>>>>>>>>>>>>>>>>>> T22
>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
------T10---------- ------T12-----
----PE-T22---------- ------PE-T12-
G-G-A-L-I-H-A-R-F-V-M-T-A-A-S-C-F-Q-S-Q-N-X-G-V-I-T-X-L-
>>>>>>>>>>>>>>>>>>>>>>>>>>
----------T12----------

A-G-T-R-C-Q-V-A-G-W-G-S-Q-R-S-G-G-R-L-S-R-F-P-R
----------PE-V10----------
F-V-N-V-T-V-T-P-E-D-Q-C-R-P-N-N-V-C-T-G-V-L-T-R-R-G-G-I-C-N
----- PE-V10 ----------
G-D-G-G-T-P-L-V-C-E-G-L-A-X-G-
----- PE-V10 ----------
G-P-D-F-F-T-X-V-A-L-F-R-D-W-I-D----G-V-L-N-N-P-G-P
----PE-T13----- --T15&PE-T30----------
-PE-T12-- ----------PE-T19------(T)
Carboxy Terminus¦

Table 1

```
          1                   10                  20
CAP37     I-V-G-G-R-K-A-R-P-R-Q-F-P-F-L-A-S-I-Q-N-Q-G-R-H-F-----
ELAST     I-V-G-G-R-R-A-R-P-H-A-W-P-F-M-V-S-L-Q-L-R-G-G-H-F---
FACTD     I-L-G-G-R-E-A-E-A-H-A-R-P-Y-M-A-S-V-Q-L-N-G-A-E-L-----
PLASM     I-V-G-G-C-V-S-K-P-H-S-W-P-W-Q-V-S-L-R-R-S-S-R-H-F-----
CATG      I-I-G-G-R-E-S-R-P-H-S-R-P-Y-M-A-Y-L-Q-I-Q-S-P-A-G-Q-
RMCPI     I-I-G-G-V-E-S-R-P-H-S-R-P-Y-M-A-H-L-E-I-T-T-E-R-G-Y-
RMCP11    I-I-G-G-V-E-S-I-P-H-S-R-P-Y-M-A-H-L-D-I-V-T-E-K-G-L-
CCPI      I-I-G-G-H-E-V-K-P-H-S-R-P-Y-M-A-L-L-S-I-K-D-Q-Q-P-E-
HF        I-I-G-G-D-T-V-V-P-H-S-R-P-Y-M-A-L-L-K-L-S-S-N-T------

                                          *
CAP37     -------C-G-G-A-L-I-H-A-R-F-V-M-T-A-A-S-C-   42
ELAST     -------C-G-A-T-L-I-A-P-N-F-V-M-S-A-A-H-C-   42
FACTD     -------C-G-G-V-L-V-A-E-Q-W-V-L-T-A-A-H-C-   42
PLASM     -------C-G-G-T-L-I-S-P-K-W-V-L-T-A-A-H-C-   42
CATG      S-R---C-G-G-F-L-V-R-E-D-F-V-L-T-A-A-H-C-   45
RMCP1     K-A-T-C-G-G-F-L-V-N-R-N-F-V-M-T-A-A-H-C-   46
RMCPII    R-V-I-C-G-G-F-L-I-S-R-Q-F-V-L-T-A-A-H-C-   46
CCPI      A---I-C-G-G-F-L-I-R-E-D-F-V-L-T-A-A-H-C-   45
HF        ----I-C-A-G-A-L-I-E-K-N-W-V-L-T-A-A-H-C-   42
```

Table 2

```
                                                                  CC          2

ATCTGTGCTAGGGCCCGGCTGCCAGGGCAGAACTCAGACTTAAAGCACAGAGAAG                     57

GCAAGCGGCTTGGCCTGGGTCACACAGCCAGCCCGGCCTGGACGATCCCGCGAAA                     112

GGCGTGAGGGCGGACGGTGTGCGGGACTCAGGGGCCCCCCTGTCCTCTTAGGGAG                     168

TCGGACG ATG GGG GAG GGT GGG TCC CCC CGC AGC CCC ACT GGG                       211
        MET Gly Glu Gly Gly Ser Pro Arg Ser Pro Thr Gly
        -48                                         -37
TGG ATA GAG CTG AGG CTG CAG CTT CAC ACC CCC TCC CGG CCA                       253
Trp Ile Glu Leu Arg Leu Gln Leu His Thr Pro Ser Arg Pro
                                                    -23
CTG TGT GGA TTC TTG GGG ATC TCA GAG CTG TCT CCC CCC GAC                       295
Leu Cys Gly Phe Leu Gly Ile Ser Glu Leu Ser Pro Pro Asp
                                                    -9
CCA GGC TCC AGC CCC CTT TTG GAC ATC GTT GGC GGC CGG AAG                       337
Pro Gly Ser Ser Pro Leu Leu Asp Ile Val Gly Gly Arg Lys
                                1                   6
GCG AGG CCC CGC CAG TTC CCG TTC CTG GCC TCC ATT CAG AAT                       379
Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln Asn
                                                    20
CAA GGC AGG CAC TTC TGC GGG GGT GCC CTG ATC CAT GCC CGC                       421
Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg
                                                    34
TTC GTG ATG ACC GCG GCC AGC TGC TTC CAA ACC CAG AAC CCC                       463
Phe Val MET Thr Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro
                                                    48
GGG GTT AGC ACC GTG GTC CTG GGT GCC TAT GAC CTG AGG CGG                       505
Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg
                                                    62
CGG GAG AGG CAG TCC CGC CAG ACG TTT TCC ATC AGC AGC ATG                       547
Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser MET
                                                    76
AGC GAG AAT GGC TAC GAC CCC CAG CAG AAC CTG AAC GAC CTG                       589
Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
                                                    90
ATG CTG CTT CAG AGG TTT GTC AAC GTG ACT GTG ACC CCC GAG                       631
MET Leu Leu Gln Arg Phe Val Asn Val Thr Val Thr Pro Glu
                                                    104
GAC CAG TGT CGC CCC AAC AAC GTG TGC ACC GGT GTG CTC ACC                       673
Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
                                                    118
CGC CGC GGT GGC ATC TGC AAT GGG GAC GGG GGC ACC CCC CTC                       715
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu
                                                    132
GTC TGC GAG GGC CTG GCC CAC GGC GTG GCC TCC TTT TCC CTG                       757
Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu
                                                    146
GGG CCC TGT GGC CGA GGC CCT GAC TTC TTC ACC CGA GTG GCG                       799
Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala
                                                    160
CTC TTC CGA GAC TGG ATC GAT GGT GTT CTC AAC AAC CCG GGA                       841
Leu Phe Arg Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly
                                                    174
CCG GGG CCA GCC TAG GGGGGCCTGTGACCTCCCATGGAGCCCAGCCCCGC                      891
Pro Gly Pro Ala End
          178
CCTCCACACCTCCGGCGCTCCGCACCCACCTCCCACGGCCCCCGCCCCTGCCCCCG                     946

TCCGGCCAGAGGGGCCCTGGCTGTAATAAAGAAGCCGATCTCTCCTCTGAAA                         998
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Table 3

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CCC ATG ACC CGG CTG ACA GTC CTG GCC CTG CTG GCT GGT CTG   42
    MET Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu
    -26                                              -14

CTG GCG TCC TCG AGG GCC GGC TCC AGC CCC CTT TTG GAC ATC   84
Leu Ala Ser Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp Ile
                                                     1

GTT GGC GGC CGG AAG GCG AGG CCC CGC CAG TTC CCG TTC CTG  126
Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
                                                    15

GCC TCC ATT CAG AAT CAA GGC AGG CAC TTC TGC GGG GGT GCC  168
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala
                                                    29

CTG ATC CAT GCC CGC TTC GTG ATG ACC GCG GCC AGC TGC TTC  210
Leu Ile His Ala Arg Phe Val MET Thr Ala Ala Ser Cys Phe
                                                    43

CAA AGC CAG AAC CCC GGG GTT AGC ACC GTG GTG CTG GGT GCC  252
Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala
                                                    57

TAT GAC CTG AGG CGG CGG GAG AGG CAG TCC CGC CAG ACG TTT  294
Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe
                                                    71

TCC ATC AGC AGC ATG AGC GAG AAT GGC TAC GAC CCC CAG CAG  336
Ser Ile Ser Ser MET Ser Glu Asn Gly Tyr Asp Pro Gln Gln
                                                    85

AAC CTG AAC GAC CTG ATG CTG CTT CAG CTG GAC CGT GAG GCC  378
Asn Leu Asn Asp Leu MET Leu Leu Gln Leu Asp Arg Glu Ala
                                                    99

AAC CTC ACC AGC AGC GTG ACG ATA CTG CCA CTG CCT CTG CAG  420
Asn Leu Thr Ser Ser Val Thr Ile Leu Pro Leu Pro Leu Gln
                                                   113

AAC GCC ACG GTG GAA GCC GGC ACC AGA TGC CAG GTG GCC GGC  462
Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala Gly
                                                   127

TGG GGG AGC CAG CGC AGT GGG GGG CGT CTC TCC CGT TTT CCC  504
Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro
                                                   141

AGG TTT GTC AAC GTG ACT GTG ACC CCC GAG GAC CAG TGT CGC  546
Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln Cys Arg
                                                   155

CCC AAC AAC GTG TGC ACC GGT GTG CTC ACC CGC CGC GGT GGC  588
Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly
                                                   169

ATC TGC AAT GGG GAC GGG GGC ACC CCC CTC GTC TGC GAG GGC  630
Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly
                                                   183

CTG GCC CAC GGC GTG GCC TCC TTT TCC CTG GGG CCC TGT GGC  672
Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly
                                                   197

CGA GGC CCT GAC TTC TTC ACC CGA GTG GCG CTC TTC CGA GAC  714
Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp
                                                   211

TGG ATC GAT GGT GTT CTC AAC AAC CCG GGA CCG GGG CCA GCC  756
Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala
                                                   225

TAG GGGGGCCTGTGACCTCCCATGGAGCCCAGCCCCGCCCTCCACACCTCCGGC  810
End

GCTCCGCACCCACCTCCCACGGCCCCGCCCCTGCCCCCGTCCGGCCAGAGGGGCC  865

CTGGCTGTAATAAAGAAGCCGATCTCTCCTCTGAAAAAAA                 905
```

Table 4

Peptide 1, F-Q-S-Q-N-P-G-V-S-T-V (Amino acids 43 - 53)

Peptide 2, S-I-S-S-M-S-E-N-G (Amino acids 72 - 80)

Peptide 3, S-Q-H-S-G-G-R-L-S-R-F-P-R-F (Amino acids 130 - 143)

Table 5

EFFECT OF PEPTIDE #1 ON MONOCYTE CHEMTAXIS

chemoattractant conc	Distance migrated in μm
10^{-7}M peptide #1	57.2
10^{-8}M peptide #1	51.5
10^{-9}M peptide #1	57.7
10^{-10}M peptide #1	63.5
10^{-11}M peptide #1	51.3
10^{-12}M peptide #1	50.2
10^{-8}M FMLP	86.2
10^{-9}M CAP37	78.4
Geys buffer	45.8

Table 6

EFFECT OF PEPTIDE #3 ON MONOCYTE CHEMOTAXIS

chemoattractant conc	Distance migrated in μm
10^{-8}M peptide #3	46.5
10^{-9}M peptide #3	52.0
10^{-10}M peptide #3	66.6
10^{-11}M peptide #3	52.8
10^{-12}M peptide #3	50.5
10^{-13}M peptide #3	50.2
10^{-8}M FMLP	91.4
10^{-9}M CAP37	73.6
Geys buffer	43.9

Table 7

IT IS CLAIMED:

1. A recombinant DNA molecule comprising a DNA sequence coding for a cationic granule protein, said protein comprising the sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro.

2. The DNA molecule of claim 1, which further encodes an amino terminal extension of said protein.

3. The DNA molecule of claim 2, where the amino terminal extension of said protein is as follows:
Met Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu Leu Ala
Ser Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp.

4. The DNA molecule of claim 2, where the amino terminal extension of said protein is as follows:
Met.

5. The DNA molecule of claim 1, which further encodes a carboxy terminal extension of said protein.

6. The DNA molecule of claim 5, where the carboxy terminal extension of said protein is as follows:
Gly Pro Ala.

7. A recombinantly produced cationic granule protein comprising the sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro.

8. The protein of claim 7, which further comprises the following amino terminal extension of said protein:
Met Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu Leu Ala
Ser Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp.

9. The protein of claim 7, which further comprises the following amino terminal extension of said protein:
Met.

10. The protein of claim 7, which further comprises the following carboxy terminal extension of said protein:
Gly Pro Ala.

11. The protein of claim 7, which further includes, attached to the amino or the carboxy terminus of the sequence, a second protein coding sequence.

12. The protein of claim 11, wherein the second protein chosen from among the group consisting of ricin A chain, abrin A chain and trichosanthin.

13. The protein of claim 7, which is a chemoattractant for monocytes.

14. The protein of claim 7, which binds to bacterial lipopolysaccharide.

15. Peptides having at least 5 amino acids which are chemotactic for monocytes and are derived from the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala.

16. Peptides having at least 5 amino acids which bind bacterial lipopolysaccharide and are derived from the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala.

17. A peptide of Claim 15 comprising at least the following amino acid sequence: -Phe-Gln-Ser-Gln-Asn-Pro-Gly-Val-Ser-Thr-Val-.

18. Peptides which are chemotactic for monocytes and which are comprised of at least the following amino acid sequence: -Ser-Gln-His-Ser-Gly-Gly-Arg-Leu-Ser-Arg-Phe-Pro-Arg-Phe-.

19. A process for obtaining a homogeneously pure, monoctye chemotactic protein from polymorphonuclear leukocytes having cell membranes, comprising the steps of:

a) disrupting the cell membranes to release granules and other intracellular components;

b) separating a granule fraction by density;

c) making a crude granule extract from said granule fraction;

d) binding said crude granule extract to a cation exchange matrix;

e) eluting said bound portion of said crude granule extract from said matrix to produce an eluate;

f) concentrating said eluate;

g) applying said concentrated eluate to a second matrix capable of separating proteins on the basis of molecular size;

h) collecting a second eluate from said second matrix;

i) fractioning said second eluate by high performance liquid chromatography though a column to produce first and second protein fractions; and

j) collecting said second fraction containing the protein having monocyte chemotactic activity.

20. The process of Claim 19 wherein said column of said fractionating step comprises a matrix that promotes hydrophobic, nondenaturing interactions with proteins.

21. A recombinant process for the production of bioactive peptides comprising,

(a) an expression vector containing a DNA sequence encoding a peptide having at least 5 amino acids which are chemotactic for monocytes and are derived from the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val

Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro,

and, said vector further containing transcriptional and translational control elements flanking said DNA sequence effective to express said DNA coding sequence,

said vector also containing a gene which facilitates selection of the vector in a suitable host;

(b) introducing said expression vector into a suitable host;

and,

(c) isolating the bioactive peptide expressed by the vector.

22. The process of claim 21, where the expression vector further includes a DNA sequence encoding a collagenase cleavage site and the β-galactosidase protein, resulting in an in-frame fusion between β-galactosidase protein, the collagenase cleavage site, and the coding sequence of the bioactive peptide.

23. The process of claim 22, where the fusion protein is isolated by affinity chromatography for the β-galactosidase protein portion of the fusion protein, and the bioactive peptide is liberated from the fusion protein by digestion of the fusion protein with the collagenase proteinase.

24. An expression vector for introduction into a host cell comprising,

a DNA sequence encoding the following protein sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro,

and,

flanking said DNA sequence, transcriptional and translational control elements effective to express said DNA coding sequence,

said vector also containing a gene which facilitates selection of the vector in the host cell.

25. The expression vector of claim 24, where the transcriptional control elements include a promoter and a poly-adenylation signal.

26. The expression vector of claim 25, where the promoter is obtained from cytomegalovirus and the polyadenylation signal is obtained from SV40.

27. The expression vector of claim 26, where the gene to facilitate selection is the dihydrofolate reductase gene.

28. A recombinant process for the production of a cationic granule protein comprising,

(a) an expression vector containing a DNA sequence encoding a protein that is comprised of the following amino acid sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro,

and, said vector further containing transcriptional and translational control elements flanking said DNA sequence effective to express said DNA coding sequence,

said vector also containing a gene which facilitates selection of the vector in a suitable host;

(b) introducing said expression vector into a suitable host;

and,

(c) isolating the recombinant protein expressed by the vector.

29. The process of claim 28, where the expression vector further includes a DNA sequence encoding a thrombin cleavage site and the glutathione-S-transferase protein (Sj26), resulting in an in-frame fusion between Sj26, the thrombin cleavage site, and the coding sequence of the cationic granule protein.

30. The process of claim 29, where the fusion protein is isolated by affinity chromatography for the Sj26 protein portion of the fusion protein, and the cationic granule protein is liberated from the fusion protein by digestion of the fusion protein with the thrombin proteinase.

31. The process of claim 28, wherein the host is selected from the group consisting of: bacteria, yeast, and mammalian cells.

32. A method of treating a wound by the application of a topical medication comprising,

a cationic granule protein comprising the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg

Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro,

and, said protein present in the medication in a pharmacologically effective amount to promote wound healing.

33. The method of claim 32, wherein said topical medication further comprises epidermal growth factor in a pharmacologically effective amount to promote wound healing.

34. The method of claim 32, wherein the form of the topical medication is selected from the group consisting of: pastes, gels, creams, and ointments.

35. A method for treating diseases involving monocyte localization or defects of monocyte chemotaxis in an animal comprising the step of administering to said animal, a therapeutic composition comprising a pharmacologically effective amount of a monocyte chemotactic protein characterized by a molecular weight of 36,000 to 38,000 daltons as measured by SDS-PAGE, under reducing conditions.

36. The method of Claim 35, further comprising, a pharmaceutically acceptable carrier.

37. A method for treating diseases in animals involving monocyte localization or defects of monocyte chemotaxis comprising the step of administering to said animal, a therapeutic composition comprising a pharmacologically effective amount of a peptide having at least 5 amino acids derived from the following sequence: Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val

Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala.

38. A method for increasing the migration of monocytes to a particular tissue site in an animal, comprising the step of administering to said tissue site a therapeutic composition, comprising a pharmacologically effective amount of a monocyte chemotactic protein.

39. The method of Claim 38, wherein said monocyte chemotactic protein is characterized by a molecular weight of between 36,000 and 38,000 daltons as measured by SDS-PAGE, under reducing conditions.

40. The method of Claim 38, wherein said monocyte chemotactic protein is comprised of a peptide having at least 5 amino acids derived from the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val

Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala.

41. A hybrid protein comprising the cationic granule protein having the following sequence:

Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu
Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser
Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu
Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly
Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly
Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr
Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val
Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro
Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg
Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro,

where said cationic protein is covalently linked to a second protein.

42. The hybrid protein of claim 41, wherein the second protein is an antibody.

43. The hybrid protein of claim 41, wherein the second protein is chosen from among the group consisting of ricin A chain, abrin A chain, and trichosanthin.

44. A homogeneously pure monocyte chemotactic protein.

45. The chemotactic protein of Claim 44, wherein said protein is purified from polymorphonuclear leukocytes, is devoid of other contaminating granule proteins including the defensins, cathepsin G, myeloperoxidase, lactoferrin, and CAP 57, and further, wherein said protein has a molecular weight as determined by SDS-PAGE, under reducing conditions, between 36,000 and 38,000 daltons.

46. The chemotactic protein of Claim 44, wherein said protein is devoid of contaminating granule proteins including the defensins, cathepsin G, myeloperoxidase, lactoferrin, and CAP57.

47. The chemotactic protein or Claim 46, wherein said protein has a molecular weight, as determined by SDS-PAGE, under reducing conditions, between 36,000 and 38,000 daltons.

48. The chemotactic protein of Claim 44, wherein said protein has a molecular weight, as determined by SDS-PAGE, under reducing conditions, between 36,000 and 38,000 daltons.

49. The chemotactic protein of Claim 46, wherein said protein has an amino terminal amino acid sequence comprising the following amino acids:

Ile-Val-Gly-Gly-Arg-Lys-Ala-Arg-Pro-Arg-Gln-Phe-Pro-Phe-Leu-Ala-Ser-Ile-Gln-Asn-Gln-Gly-Arg-His-Phe-Cys-Gly-Gly-Ala-Leu-Ile-His-Ala-Arg-Phe-Val-Met-Thr-Ala-Ala-Ser-Cys-Phe-Gln-Ser-Gln-Asn-

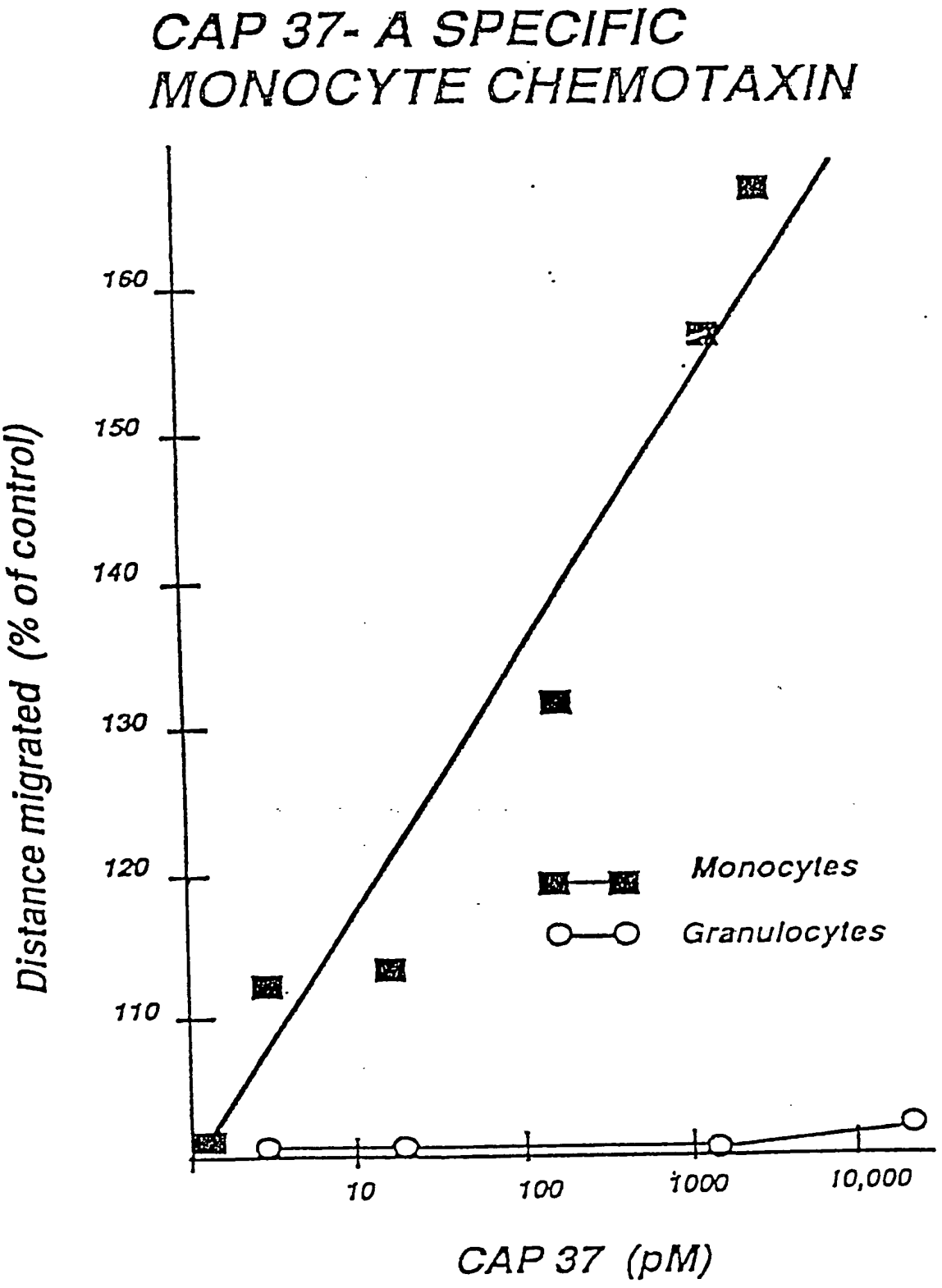
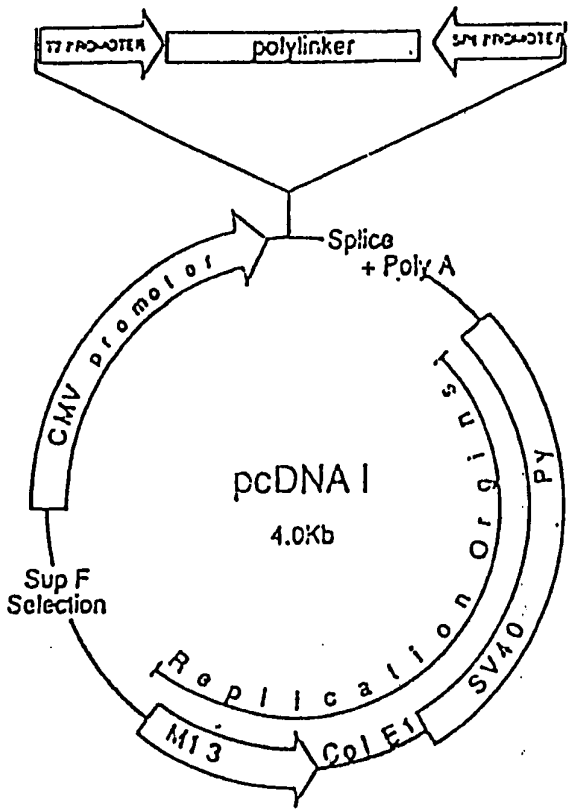


FIG.1



T7 PRIMER ——>

CMV/T7 PROMOTERS ——>+1 . HindIII KpnI SacI BamHI SpeI XmaIII BstXI

CTATAGTGAGTCGTATTAGAGACCGGAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTG

EcoRI PstI EcoRV BstXI NotI XhoI SphI XbaI ApaI .+1 <—— Sp6 PROMOTER

GAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCTATAGTGTCACCTAAAT

<—— Sp6 PRIMER

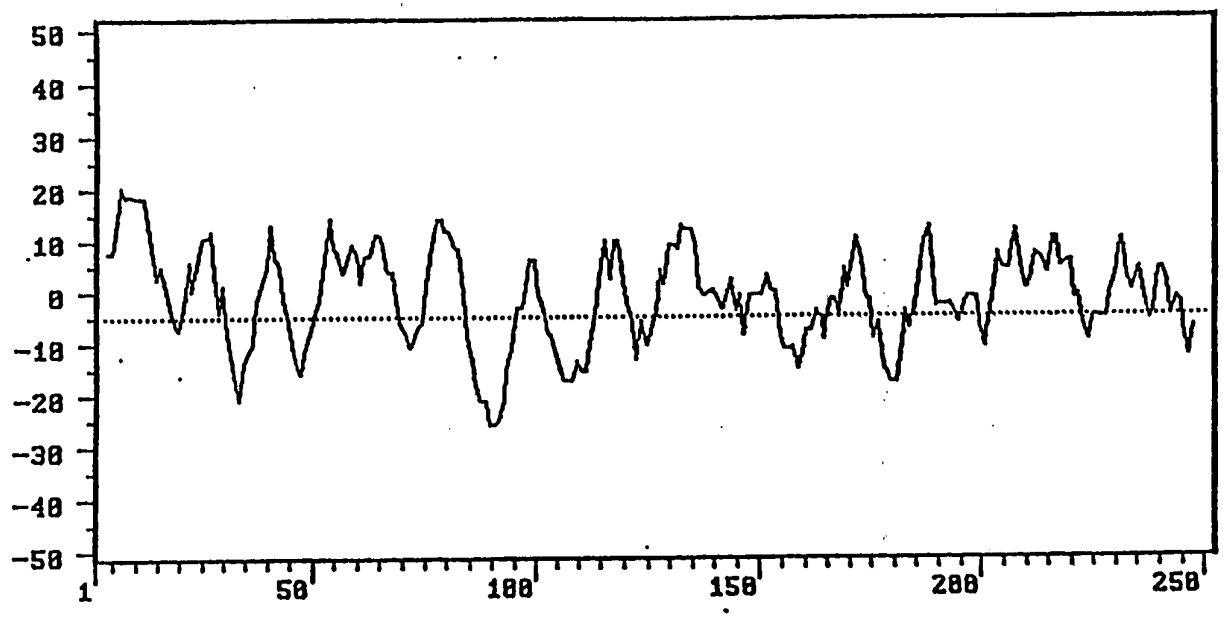


FIG. 3

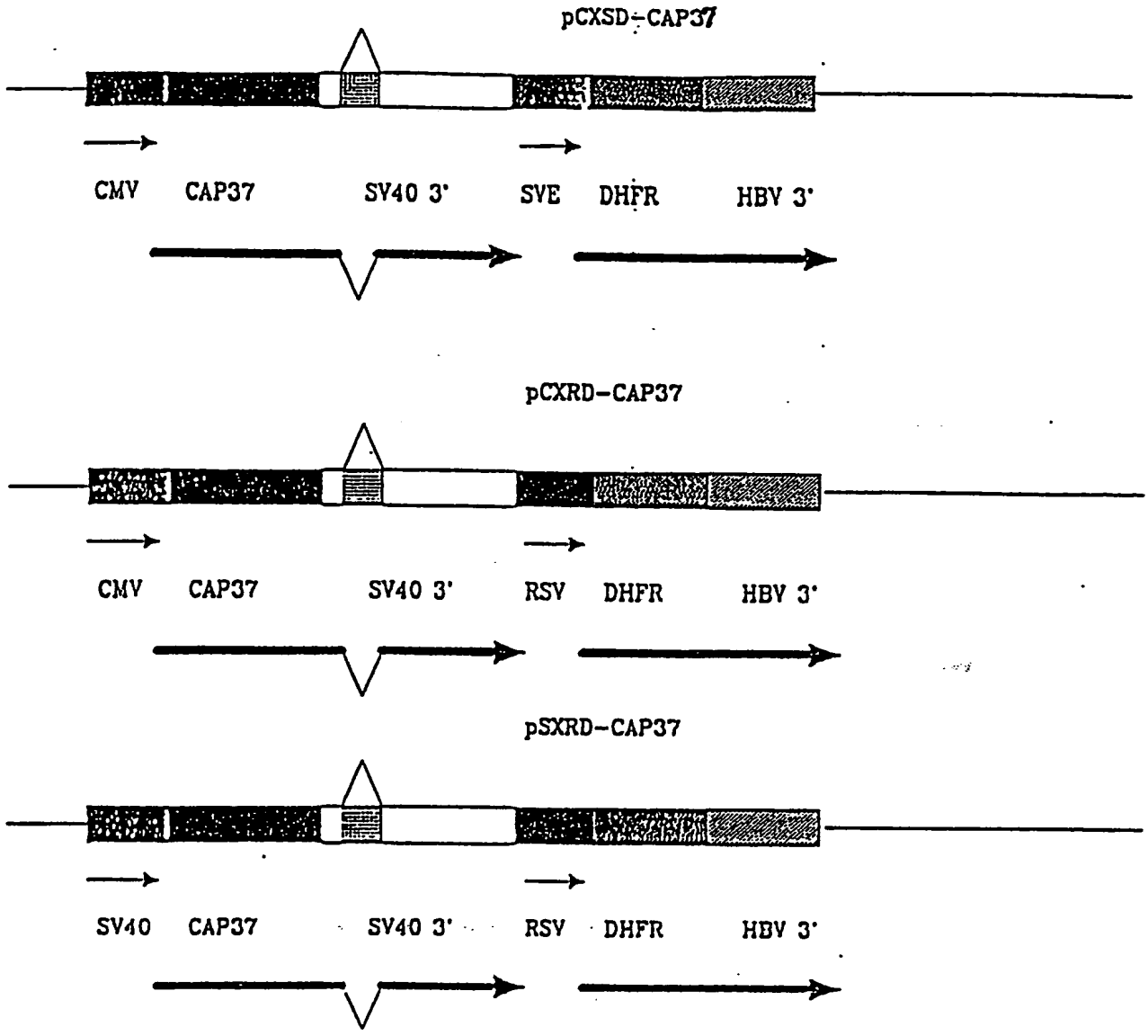


FIG. 4

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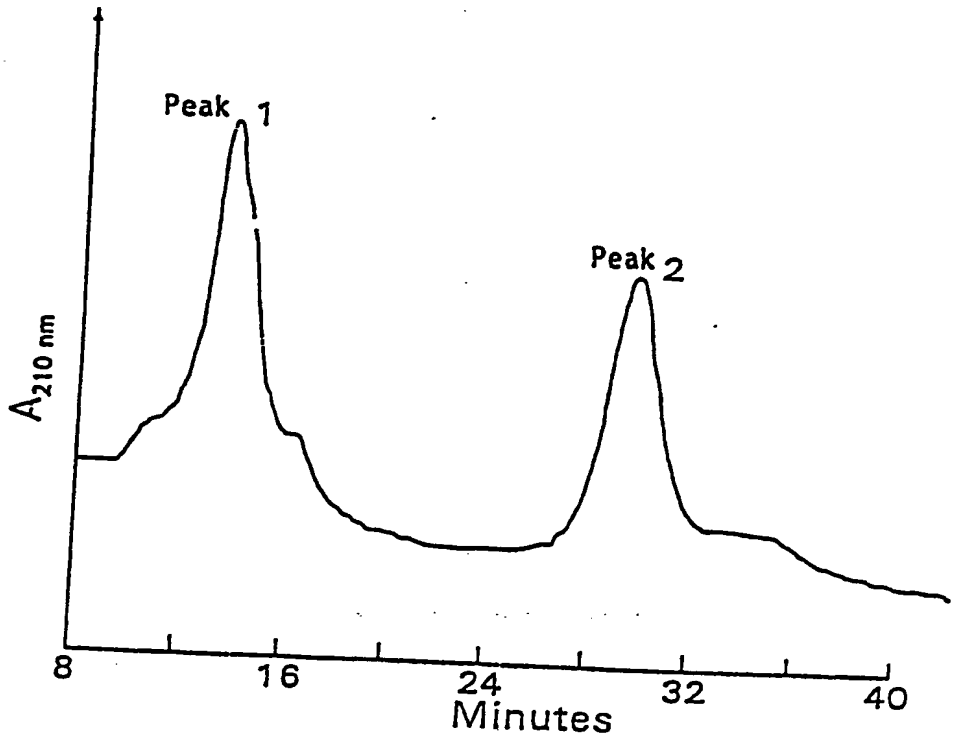

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90?/03772

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/09,15/11,15/19,15/63; C07K 3/18,3/22,3/28,7/06,7/08,7/10,7/40,15/02,15/26

U.S.Cl : 530/351,415,416,417,300,402,427,330,327; 514/17,21; 435/172.3,320; 536/27

II. FIELDS SEARCHED [4]

Minimum Documentation Searched [4]

Classification System [5]	Classification Symbols
U.S.Cl.	530/351,415,416,417,427,330; 514/17,21; 435/172.3,320; 536/27 530/402,327,300

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

SEARCHED US PAT AND DIALOG FILES 5,35,72,340,350,351,357 and 399 and SEQUENCES IN PIR, SWISS PROT, EMBL. SEARCHED FOR CHEMOTATIC ACTIVITY WITH MW OR MONOCYTE AND METHODS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category * [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X Y A	Infection and Immunity, Vol. 53, No. 3, issued September 1986, (Shafer et al.), "Late Intraphagosomal Hydrogen Ion Concentration Favors the In Vitro Antimicrobial Capacity of a 37-Kilodalton Cationic Granule Protein of Human Neutrophil Granulocytes", pages 651-655. See pages 651-653.	7,9,44-49 1-6,8,10-18,21-31 19,20
X,P Y	The Journal of Clinical Investigation, Vol. 85, issued May 1990, (Pereira et al.), "CAP37, a Human Neutrophil-Derived Chemotactic Factor with Monocyte Specific Activity", see pages 1468-1476.	7,9,44-49 7, 1-6,8,10-30
X Y	Cellular Immunology, Vol. 26, No. 2, issued 1976, (Leonard et al.), "Characterization of Mouse Lymphocyte Derived Chemo Tactic Factor", Abstract, BIOSIS No. 63020997, see pages 200-210.	7,44-49 7,9,44-49

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [1]
25 September 1990	06 DEC 1990
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Keith C. Furman

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No **
Y	Gene, Vol. 67, issued 1988, (Smith et al.), "Single-Step Purification of Polypeptides Expressed in Escherichia Coli as Fusions with Glutathione S-Transferase", pages 31-40. See pages 31 and 38.	28-31
Y	US, A, 4,543,329 (Daum et al.), 24 September 1985, see the abstract, column 1 and column 9.	10,21-24
Y	The Journal of Biological Chemistry, Vol.263, No. 15, issued May 1988, (Hanada et al.) "Purification and Reconstitution of Escherichia coli Proline Carrier Using a Site Specifically Cleavable Fusion Protein", pages 7181-7185. See pages 7181 and 7182.	10,21-24
X,P	Chemical Abstracts No. 111:34668z, Published 1990, (Tsmo et al.), "An Episomal Vector for the Expression of Selected DNA Fragments Coding for Polypeptides in Mammalian Cells and Methods for the Production Thereof", European Patent Application EP 292,879, 30 November 1988.	24-27
Y	Chemical Abstracts No. 110:207240p, Published 1989, Piatak et al. "Recombinant Ricin B Muteins and Their use in the Preparation of Conjugates of Ricin muteins with Binding Proteins Such as Antibodies", PCT International Application WO 88 07,081, 22 September 1988. See abstract	11,12,41-43
Y,P	US, A, 4,912,111 (Sank et al.), 27 March 1990, see columns 3 and 4.	32-34
Y	Abstract, WPI Accession No 86-046523/07 of JP 61001616 (Teijin), 07 January 1986. "Monocyte Chemotactic factor has activity for human blood monocyte". See abstract.	44

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	Abstract, BIOSIS No. 88054394 of The International Journal of Biochemistry, Vol. 21, No.4, Published 1989, "Further Studies on a Chemoattractant Derived from Aleurites-Forddi Hemsl. Seed", (Schichigo et al.), pages 433-438.	7,44-49 7,9,44-49
Y	Gene, Vol. 62, issued 1988, (Scholtissek et al.) "A Plasmid Vector System for the Expression of a Triprotein Consisting of beta-Galactosidase, a Collagenase Recognition Site and a Foreign Gene Product", pages 55-64. See pages 55, 58, 63 and 64.	10,21-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter[1] not required to be searched by this Authority namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out[1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHED SHEET

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT/US90/03772
Attachment to form PCT/ISA/210 supplement sheet 2:

Specifically, the inventions are as follows:

I. Claims 1-4, 5, 6 and 21-23 drawn to a first invention comprising first product (DNA molecules) and first method of using (the recombinant processes of using the DNA molecules)- NOTE- claims 5 and 6 are a first specie of the generic molecule,

II. Claims 7-14 and 41-43 which are drawn to a second invention comprising a second product (a specific cationic granule proptein),

III. Claims 15-17 which are drawn to a third invention comprising a third product (a specific peptide fragment),

IV. Claim 18 which is drawn to a fourth invention comprising a fourth product (specific peptide fragment of 14 amino acids),

V. Claims 19 and 20 drawn to a fifth invention comprising a second process (method of purifying proteins in general),

VI. Claims 24-27 drawn to a sixth invention comprising a fifth product (an expression vector),

VII.Claims 28-31 drawn to seventh invention comprising a third process (method of recombinantly producing a specific protein),

VIII.Claims 32-34 drawn to an eighth invention comprising a fourth process (method of treating a wound with a specific protein),

IX. Claims 35,36, and 38-40 drawn to a ninth invention comprising a fifth process (method of treating a disease with a specific protein),

X. Claim 37 drawn to a tenth invention comprising a sixth process (method of treating a disease with a specific peptide), and

XI. Claims 44-49 drawn to an eleventh invention comprising a sixth product (homogenously pure chemotactic protein).

The fee for searching of ten additional inventions is $1,500; however, the searchable claims of Group VII could be searched without effort justifying an additional fee (when the claims of Group II are searched). Therefore, the claims of Group VII can be grouped with those of Group II for the purposes of this requirement.

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